IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Mehraban et al.	Examiner:	Yao, Lei
Serial No.:	09/703,350	Group Art Unit:	1642
Filed:	October 31, 2000	Docket No.:	11669.213USU1
Customer No.:	23552	Confirmation No.:	3065
Tech Center:	Biotechnology and Organic Chemistry (1600)		
Title:	DIFFERENTIALLY EXPRESSED GENES INVOLVED IN ANGIOGENESIS, THE POLYPEPTIDES ENCODED THEREBY, AND METHODS OF USING THE SAME		

Filed EFS-WEB

APPELLANT'S BRIEF ON APPEAL

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

23552
PATENT TRADEMARK OFFICE

Sir:

This Brief is presented in support of the Appeal filed May 20, 2008, from the final rejection of Claims 56 and 69-79 of the above-identified application, as set forth in the Final Office Action mailed February 20, 2008. Payment in the amount of $510 to cover the required fee for filing this Brief is being submitted via credit card.

An oral hearing is requested. A separate request for oral hearing with the appropriate fee will be filed within two months of the Examiner's Answer.

TABLE OF CONTENTS

I. REAL PARTY OF INTEREST

The real party in interest for this appeal is Genentech Inc. (hereinafter "Applicant"), the assignee of record. An Assignment recorded on August 24, 2001 at Reel 012104, Frame 0764 lists Genentech Inc as the assignee of the present application.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF CLAIMS

Claims 56 and 69-79 are pending. Claims 1-55, 57-68, and 80 are cancelled. No claims are allowed. No claims are objected to. Claims 56 and 69-79 are rejected. Claims 56 and 69-79 are being appealed.

IV. STATUS OF AMENDMENTS

No amendment was filed following the Final Rejection mailed on February 20, 2008.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

Stanniocalcin is a secreted glycoprotein that was known to be expressed in kidney and thymic stromal cells and reported to be involved in calcium and phosphate regulation. Stanniocalcin has an amino acid sequence of SEQ ID NO:76 and is encoded by a polynucleotide having the nucleotide sequence of SEQ ID NO:75.

Applicants disclose a working example showing upregulation of stanniocalcin precursor in an art recognized model for angiogenesis. *See* Example 19 in the specification at page 142 and Fig. 23. Stanniocalcin precursor expression was found to be dramatically enhanced under tube-forming conditions, demonstrating a strong correlation between expression of stanniocalcin and tube-formation. *See* the specification at page 25, lines 20-26 and Example 19 at page 142. Applicants also disclose that stanniocalcin is expressed in ductal mammary adenocarcinoma, squamous cell carcinoma, chrondosarcoma, and renal cell carcinoma vasculature but not in normal vessels. *See* the specification at page 145, line 32 to page 146, line 7 and Figures 28 and 29. The combination of increased expression in endothelial cells in a model for angiogenesis, and increased expression in tumor tissue provides a correlation of the relationship of upregulation of stanniocalcin with angiogenesis in tumor tissues and indicating that antagonists of stanniocalcin would be useful for inhibiting tumor angiogenesis.

The pending claims include one independent claim summarized below. Claims depending from the independent claim, where argued separately, are also summarized. Citation to portions of the supporting description found in the specification is provided for the benefit of the Board.

Independent claim 56 is directed to a method for inhibiting angiogenesis in a tumor by administering to a tumor an effective amount of an antibody or antigen binding fragment of the antibody that specifically binds a polypeptide comprising an amino acid sequence of SEQ ID NO:76 and inhibits or neutralizes said polypeptide.

The amino acid sequence of SEQ ID NO:76 corresponds to PA23, which is defined in the specification at Table 1 on page 11 and page 25, lines 10-14 as a stanniocalcin precursor with GenBank accession number of U25997. In the non-final Office Action mailed on March 5, 2004, the Examiner required Applicants to amend the disclosure to include the amino acid sequence of PA23, which was incorporated by reference in the specification as originally filed.

In the Amendment and Response filed on September 2, 2004, Applicants amended the claims, sequence listing, and disclosure to include the nucleotide (SEQ ID NO:75) and amino acid (SEQ ID NO:76) sequence for stanniocalcin precursor.

As required by MPEP §608.01(p), a declaration stating that the amendatory material consists of the same material incorporated by reference in the referencing publication was filed with the Amendment and Response on September 2, 2004. GenBank accession number U25997 corresponds to the nucleic acid sequence of SEQ ID NO:75. SEQ ID NO:76 was known in the art and published in Olsen *et al.*, *Human Stanniocalcin: a Possible Hormonal Regulator of Mineral Metabolism* 93 Proc. Nat'l Acad. Sci. *USA* 1792 (1996) (cited in the specification at page 25, line 12).

Support for inhibiting angiogenesis in a tumor is found in the specification, for example, at page 105, lines 27-30 and page 107, lines 19-32. Support for administering an antagonist of a PA polypeptide to the tumor is found throughout the specification including at page 12, lines 25-28, page 52, lines 4 and 10-14, page 105, lines 27-30, page 107, lines 19-32, and page 115, lines 10-12. Support for PA23 and stanniocalcin precursor as a PA polypeptide is found at least in the specification at Table 1 on page 11, page 25, line 10, and page 30, lines 5-14. Support for an antagonist that inhibits or neutralizes a PA polypeptide is found throughout the specification including at page 25, lines 17-19 and page 40, lines 12-14. Support for an antagonist antibody or antibody binding fragment thereof is found in the specification at least at page 38, lines 12-18, page 40, lines 16-17, and page 41, lines 28-32. Support for effective amount of an antagonist of a PA polypeptide is found in the specification including at page 47, lines 29-31 and page 114, lines 3-9 and 17-19.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Whether claims 56 and 69-79 lack enablement and are, therefore, unpatentable under 35 U.S.C. § 112, first paragraph.

VII. ARGUMENT

In the Final Office Action mailed February 20, 2008, the Examiner rejected claims 56 and 69-79 under 35 U.S.C. § 1123, first paragraph, as failing to comply with the enablement requirement. Applicants submit the claims are enabled by the specification for the reasons discussed herein.

The following arguments are applicable to independent claim 56 unless specifically addressed to a particular claim. As dependent claims 69-79 depend from claim 56, the following arguments are also applicable to all of the dependent claims unless specifically addressed to a particular claim.

Introduction

The Examiner argues in the Office Action dated April 25, 2006, again in the Final Office Action dated October 13, 2006, again in the Office Action dated June 28, 2007, and again in the Final Office Action dated February 28, 2008 that the specification does not provide any guidance or objective evidence that inhibiting or neutralizing stanniocalcin in a mammal or tumor would effectively inhibit angiogenesis. The Examiner further argues in the Office Action dated April 25, 2006, again in the Final Office Action dated October 13, 2006, again in the Office Action dated June 28, 2007, and again in the Final Office Action dated February 28, 2008 that objective evidence such as a working example is necessary to enable one skilled in the art to make and/or use the claimed invention. Applicants respectfully do not agree.

There are many factors to be considered in an analysis of enablement, including breadth of the claims, nature of the invention, the state of the prior art, the level of ordinary skill, level of predictability in the art, the amount of direction provided by the inventor and the existence of working examples, and the quantity of experimentation. United States Patent & Trademark Office, Manual of Patent Examining Procedure § 2164.01(a) (hereinafter MPEP) (citing *In Re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988)). Only a reasonable correlation between the specification and the scope of enablement is required. MPEP § 2164.02 (citing *Cross v. Iizuka*, 753 F.2d 1040, 1050 (Fed. Cir. 1985)) (emphasis added). If all the other factors point toward enablement, the lack of working examples will not by itself render the claimed invention non-enabled. MPEP § 2164.02.

An enabling disclosure only requires a reasonable correlation to the scope of the claims. The Examiner's apparent position that the specification cannot teach how to use the claimed method unless it provides a working example that provides objective evidence that inhibiting stanniocalcin in a tumor would effectively inhibit angiogenesis is contrary to controlling case law. *See, e.g., In re Brana*, 51 F.3d 1560, 1568 (Fed. Cir. 1995). In *Brana*, the claims were directed to compounds disclosed as anti-cancer agents. *Id.* at 1562. The USPTO rejected the claims as nonenabled, *id.* at 1563-64, despite working examples in Brana's specification showing treatment of cancer in a mouse model. *Id.* at 1562-63. The *Brana* court held that "[u]sefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans." *Id.* at 1568. Therefore, whether the treatment will prove to be effective in humans is not a reasonable standard by which to measure enablement.

An example is not necessary if the invention is otherwise disclosed in such a manner that one skilled in the art would be able to practice it without an undue amount of experimentation MPEP § 2164.02. A substantial amount of experimentation is permissible if the experimentation is routine or if the specification provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Wands*, 858 F.2d at 737 (emphasis added); *see also In re Angstadt*, 537 F.2d 498, 502 (C.C.P.A. 1976). The mere fact that that the experimentation may be difficult and time consuming does not mandate a conclusion that such experimentation would be considered undue, as great expenditures of time and effort may ordinarily be employed in the field. *Falko-Gunter vs. Inglis,* 448 F.3d 1357, 1367 (Fed. Cir. 2006).

Moreover, a claim does not lack enablement merely because it encompasses inoperative embodiments. *Atlas Powder Co. v. E.I. DuPont De Nemours & Co.*, 750 F.2d 1569, 1576 (Fed. Cir. 1984). Thus, claims to a method do not lack enablement merely because a difficult-to-achieve outcome is encompassed by the claims. *See In re Cortright*, 165 F.3d 1353, 1359 (Fed. Cir. 1999) (claims encompassing achieving full head of hair held enabled by evidence showing three-fold increase in hair number, filling-in, and fuzz).

The Examiner bears the initial burden of showing that a claimed invention is nonenabled. "[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." *In re Marzocchi*, 439 F.2d 220, 223 (C.C.P.A. 1971). "[T]he PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application." *In re Wright*, 999 F.2d 1557, 1561-62 (Fed. Cir. 1993).

Applying these standards, Applicants assert the claims are enabled by the specification and would not require undue experimentation. Applicants submit that the Examiner has not met her burden to show the claimed invention is non enabled. The standard for enablement being applied by the Examiner is much too stringent and not in accord with the case law. Even if it can be concluded that the Examiner has met the initial burden, Applicants have provided evidence that clearly establishes the enablement of the claimed subject matter and rebuts the position of the Examiner.

Argument

Claims 56 and 69-79

There are many factors to be considered in an analysis of enablement, including breadth of the claims, nature of the invention, the state of the prior art, the level of ordinary skill, level of predictability in the art, the amount of direction provided by the inventor and the existence of working examples, and the quantity of experimentation. A substantial amount of experimentation is permissible if the experimentation is routine or if the specification provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Wands*, 858 F.2d at 737 (emphasis added). A method of inhibiting angiogenesis as described by the specification is enabled, interalia, through the use of the art recognized model of angiogenesis, and demonstration of expression of stanniocalcin in the vasculature of tumor tissue.

The specification describes stanniocalcin precursor and provides an accession number for the sequence information. Applicants amended the specification to include the sequence information even though Applicants assert this information was not necessary because the information was publicly available. *Falko-Gunter vs. Inglis,* 448 F.3d at 1368. The claims are directed to a method of using an antibody that specifically binds and inhibits or neutralizes a polypeptide comprising an amino acid sequence of SEQ ID NO:76. Applicants have described making and screening antibodies that specifically bind to the polypeptide at pages 85 to 95 in the specification. Methods of making antibodies and screening antibodies for inhibition or neutralization are also known to those of skill in the art.

Applicants have provided *in vitro* and in vivo evidence that the expression of stanniocalcin precursor correlates with an increase in angiogenesis. The specification provides a working example showing upregulation of stanniocalcin precursor in endothelial cells undergoing tube formation. *See* Example 19 in the specification at page 142 and Fig. 23. The endothelial cell model for tube formation is an art recognized model for angiogenesis. Davis *et al., An α2β1 Integrin-Dependent Pinocytic Mechanism Involving Intracellular Vacuole Formation & Coalescence Regulates Capillary Lumen and Tube Formation in Three-Dimensional Collagen Matrix* 224 Experimental Cell Res.39, 39-51 (1996) (cited in IDS of September 2, 2004). The specification teaches that stanniocalcin precursor expression is dramatically enhanced under tube-forming conditions. *See* the specification at page 25, lines 20-26 and Example 19 at page 142. In contrast, lower levels of stanniocalcin precursor are expressed under conditions that do not foster tube formation. This data demonstrates a strong correlation between expression of stanniocalcin and tube-formation.

Applicants also examined tissue samples from tumor tissue and demonstrated expression of stanniocalcin precursor in the tumor vasculature but not in normal vasculature. The specification shows that stanniocalcin is expressed in ductal mammary adenocarcinoma, squamous cell carcinoma, chrondosarcoma, and renal cell carcinoma vasculature, but is not expressed in normal vessels. *See* the specification at page 145, line 32 to page 146, line 7 and Figures 28 and 29. The combination of increased expression in endothelial cells in an art accepted model for angiogenesis, and increased expression in tumor tissue provides a reasonable correlation of the relationship of upregulation of stanniocalcin with angiogenesis in tumor

tissues. Applicants therefore submit that they have provided working examples and sufficient guidance.

Applicants submit that the level of skill in this art is high and the use of antibodies in inhibiting angiogenesis is not unpredictable. The Examiner's position concerning unpredictability of treatment of cancer does not take into account that a method of inhibiting angiogenesis as described by the specification is enabled, interalia, through the use of the art recognized model of angiogenesis. The Examiner admits that HUVECS are an art recognized model of angiogenesis. *See* page 7 of the Office Action of February 20, 2008.

Applicants submit the references cited by the Examiner do not accurately reflect the state of the art of use of antibodies for inhibiting angiogenesis. At least one of the cited references is almost 20 years old. Applicants have submitted evidence that the art of administering antibodies for inhibition of angiogenesis has advanced. Antibodies that inhibit angiogenesis *in vitro* have been shown to inhibit angiogenesis *in vivo*. For example, anti-VEGF antibodies were known to inhibit angiogenesis both *in vitro* and *in vivo* (*see* Presta *et al.*, *Humanization of an Anti-Vascular Endothelial Growth Factor Monoclonal Antibody for the Therapy of Solid Tumors & Other Disorders* 57 Cancer Res. 4593, 4593-4599 (1997), cited in IDS of September 2, 2004 and July 25, 2006) and have been approved by the FDA for treating cancer (*see* Press Release, Genentech, Inc. (Feb. 26, 2004) (cited in IDS of July 25, 2006)). Methods for enhancing antibody tumor penetration and biodistribution were known at the time of filing of the present application. For example, Eccles, *Monoclonal Antibodies Targeting Cancer: 'Magic Bullets' or Just the Trigger?* 3 *Breast Can. Res.*, 86, 86-90 (2000) (cited in IDS of November 11, 2007 and July 25, 2006) discloses that antibody penetration into solid tumors can be improved by removing the constant (Fc) region and preparing monomeric or dimeric antibody fragments such as Fab, $F(ab')_2$, and scFV. Applicants describe such antibody fragments and methods for making the fragments in the specification, for example, at page 41, lines 28 to page 42, line 18, page 42, line 26 to page 43, line 5, page 88, lines 5-7 and 11-14, and page 90, line 22 to page 91, line 22.

Applicants further contend that the Examiner is requiring Applicants to establish enablement to a higher degree of certainty than is required. The significant emphasis by the Examiner on the lack of clinical efficacy and alleged inability of the specification to guarantee

success in vivo, in effect is requiring clinical data to establish enablement. In the Final Office Action of February 20, 2008 on page 5, the Examiner states:

> "Again as discussed in the rejection above: overview of monoclonal antibody therapy including some promising activity, however, major obstacles **for clinical efficacy** still exist extending the unpredictability of this treatment. This includes impaired distribution and delivery of antibody to the tumor site, inadequate trafficking of potential cellular effectors to tumor, antigenic heterogeneity, shed or internalized targets and insufficient target specificity.. . etc. Applicant has neither shown that claimed method have been experimented and successfully done, nor shown the evidence or direction indicating predictable expectation of success of claimed method, which could allow one skilled in the art to practice it without undue a quantity of experimentations."(emphasis added)

Applicants submit that the standard required for enablement is not one of clinical efficacy. As stated in *Brana*, the Federal Circuit indicated that "[t]he stage at which an invention in this field becomes useful is well before it is ready to be administered to humans." 51 F.3d at 1568.

Applicants submit that an enabling disclosure only requires a reasonable correlation to the scope of the claims and a claim does not lack enablement merely because it encompasses inoperative embodiments. *Atlas Powder*, 750 F.2d at 1576. Again, the Examiner is using an improper standard to assess enablement of the claimed subject matter. In the Final Office Action of February 20, 2008 on page 6, the Examiner states:

> "However, it does not **guarantee** to neutralize the function of the stanniocalcin protein and used for inhibiting angiogenesis in a tumor in vivo because one skilled in the art clearly know a) antibody binding to its antigenic protein would not be necessary to neutralize the function of the protein and b) tumor angiogenesis is a complicated process in which many factors or proteins are involved, blocking a function of a protein **may not inhibit or affect the entirety of the angiogenic process** in a tumor, especially in vivo."

Applicants submit the standard for enablement does not require a guarantee or that the method work to inhibit or affect the entirety of the angiogenic process. *See In re Cortright*, 165 F.3d at 1359 (claims encompassing achieving full head of hair held enabled by evidence showing three-fold increase in hair number, filling-in, and fuzz).

Moreover, Applicants submit that if any experimentation were required it would be routine experimentation. A substantial amount of experimentation is permissible if the experimentation is routine or if the specification provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Wands*, 858 F.2d at 737 (emphasis added). The mere fact that that the experimentation may be difficult and time

consuming does not mandate a conclusion that such experimentation would be considered undue, as great expenditures of time and effort may ordinarily be employed in the field. *Falko-Gunter,* 448 F.3d at 1367.

Applicants submit that they have described methods and animal models for determining the efficacy of antibodies, for example, at page 76 of the specification, lines 1-25. Other *in vitro* and *in vivo* angiogenesis methods are known to those of skill in the art. Formulations and dosage for antibodies have been described, for example, at page 77 of the specification. Additional guidance for administration and dosage of antibody treatment is known to those of skill in the art including as described in Presta *et al.*, *Humanization of an Anti-Vascular Endothelial Growth Factor Monoclonal Antibody for the Therapy of Solid Tumors & Other Disorders* 57 Cancer Res. 4593, 4593-4599 (1997) (cited in IDS of September 2, 2004 and July 25, 2006). Applicants submit one of skill in the art reading the specification combined with knowledge of administration of other antibodies or anti angiogenesis treatments would be enabled to use the claimed subject matter without undue experimentation.

Thus, Applicants submit that the Examiner has not met her burden to show the claimed invention is nonenabled. The standard for enablement being applied by the Examiner is much too stringent and not in accord with the case law. The standard for enablement does not require clinical efficacy or a guarantee. All that is required is a reasonable correlation between the specification and the claims. Applicants submit one of skill in the art reading the specification would find a reasonable correlation between angiogenesis and expression of stanniocalcin. Applicants further submit that if any experimentation is required such experimentation is routine and the specification provides guidance as to how the experimentation should proceed. Applicants request that the Board reverse the rejection of the claims on this basis.

Even if, in the unlikely event, that the Examiner is found to have met the initial burden for establishing that the claims are nonenabled, Applicants submit they have provided evidence that confirms the enablement of Applicants' claims and rebuts the position of the Examiner.

Several post filing date references confirm the correlation of expression of stanniocalcin and angiogenesis described in Applicants' specification. For example, Filvaroff *et al.* found that transgenic mice over expressing stanniocalcin had significantly higher capillary density in organs and muscles compared with age-matched wildtype littermates. *See* Filvaroff *et al.*, *Stanniocalcin*

1 Alters Muscle & Bone Structure & Function in Transgenic Mice 143 *Endocrinology* 3681, 3689, first column, third paragraph (2002) (cited in IDS of September 2, 2004 and February 1, 2006). Filvaroff *et al.* also found that stanniocalcin 1 transgenic mice showed a larger increase in vascularity after femoral ligation compared to wildtype littermates. Thus, overexpression of stanniocalcin 1 leads to an increase in vascularity *in vivo*. This data further supports involvement of stanniocalcin in angiogenesis.

Gerritsen *et al.* studied gene expression using three different *in vitro* angiogenic models:HGF and VEGF in collagen gel; PMA, VEGF, and BFGF in collagen gel; and PMA, VEGF, bFGF in fibrin gel. *See* Gerritsen *et al.*, *In Silico Data Filtering to Identify New Angiogenesis Targets From a Large* In Vitro *Profiling Data Set* 10 Physiological Genomics 13 (2002) (cited in IDS of November 27, 2007 and May 24, 2005). Stably induced genes from each of these three models were identified and compared. Stanniocalcin was identified as one of the genes whose expression was upregulated in all three models and was stably upregulated through tube formation (48 hours). *See id.* at Figure 3A. Stanniocalcin was further evaluated for its role in angiogenesis in VEGF corneal tissue and expression in VEGF treated eyes was found to be dramatically higher. *Id.* (emphasis added).

Zlot *et al.* confirm that PMA stimulates the release of stanniocalcin from endothelial cells. *See, e.g.*, Zlot *et al.*, *Stanniocalcin 1 is an Autocrine Modulator of Endothelial Angiogenic Responses to Hepatocyte Growth Factor* 278 Journal of Biological Chemistry 47654, 47655, Table 1 (2003) (cited in IDS of September 2, 2004 and February 1, 2006). The gels in the angiogenesis tube formation assay shown in the specification were supplemented with PMA and Applicants show expression of stanniocalcin was upregulated in endothelial cells undergoing tube formation. *See*, for example, the specification at page 123, line 17.

Kahn *et al.* demonstrated that stanniocalcin is upregulated in an endothelial cell tube model from the start of initial tube formation through full formation of the tubes (48 hours) as measured using the gene calling method and reverse transcriptase polymerase chain reaction. *See* Kahn *et al.*, *Gene Expression Profiling in an* In Vitro *Model of Angiogenesis* 156 The Am. J. of Pathology 1887 (2000) (cited in IDS of September 2, 2004 and February 1, 2006). Significantly, when tube formation was inhibited using a PPAR ligand, 15d-PGJ2, the expression level of stanniocalcin was decreased. *See id.* at Table 4. The correlation of a decrease in

expression with inhibition of tube formation in Kahn *et al.* is evidence in support of the contention that inhibition of angiogenesis is correlated with inhibition of stanniocalcin.

Several post filing date articles confirm an association of the upregulation of expression of stanniocalcin with tumor tissue undergoing angiogenesis. Gerritsen *et al.* compared gene expression in colon tumor samples versus normal tissue, and stanniocalcin was found to be one of the most highly upregulated genes in colon tissue. *See* Gerritsen *et al.* at Figure 3B. Stanniocalcin expression was also shown in colon adenocarcinomas by *in situ* hybridization. *See id.* at Figure 5. McCudden *et al.* demonstrated that STC-1 and its receptor co-localized in breast cancer cells in 91% of cases. *See* McCudden *et al.*, *Co-localization of Stanniocalcin-1 Ligand & Receptor in Human Breast Carcinomas* 213 Molecular Cellular Endocrinology 167 (2004) (cited in IDS of February 1, 2006 and April 11, 2007). Yeung *et al.* demonstrated stanniocalcin was induced in human tumor cells, such as colon carcinoma, nasopharyngeal cancer, and ovarian cancer cultured under hypoxic conditions. *See* Yeung *et al.*, *Hypoxia-Inducible Factor-1 in Human Activation of Stanniocalcin-1 in Human Cancer Cells* 146 Endocrinology 4951 (2005) (cited in IDS of November 27, 2007).

Wascher *et al.* demonstrated STC-1 was localized in invasive and ductal carcinoma in situ using an antibody to STC-1. STC-1 mRNA was detected in breast cancer cells by *in situ* hybridization and correlated with primary tumor size, number of positive lymph nodes, and stage of the cancer cell. *See* Wascher *et al.*, *Stanniocalcin-1: A Novel Molecular Blood & Bone Marrow Marker for Human Breast Cancer* 9 Clinical Cancer Res. 1427 (2003) (cited in IDS of April 11, 2007).

In the Office Action dated June 28, 2007 and again in the Final Office Action dated February 28, 2008, the Examiner contends that the art of inhibiting angiogenesis is highly unpredictable. The Examiner cites Mook *et al.*, 2004, *Biochim. Biophys. Acta*, 1705-69-89 as evidence of unpredictability in the art. Applicants respectfully do not agree.

Mook *et al.* is directed to an entirely different type of molecule-matrix metalloproteins (MMP). Mook discloses in preclinical animal experiments that inhibitors of MMP reduce cancer progression and metastasis. The inhibitors were not as effective in human clinical trials. However, the human trials were performed on patients with advanced stages of cancer and the animal experiments showed that MMP inhibitors are effective but only when they are

administered in early stages of tumor development. *See* Mook *et al.*, *The Role of Geltinases in Colorectal Cancer Progression & Mestasis* 1705 Biochemica Et Biophysica Acta 69, 85, second column (2004). Mook *et al.* therefore does not teach that inhibition of angiogenesis is highly unpredictable. Moreover, any teaching of unpredictability relates to MMPs and not to stanniocalcin.

In contrast, Applicants have provided evidence of the predictability of inhibition of angiogenesis using antibodies. Antibodies that inhibit angiogenesis *in vitro* have been shown to inhibit angiogenesis *in vivo*. For example, several anti-VEGF antibodies were known to inhibit angiogenesis both *in vitro* and *in vivo* and have been approved by the FDA for treating cancer. The success of anti-angiogenesis agents, including antibody antagonists, in treating cancer prompted the FDA commissioner to state that antiangiogenesis therapy is the fourth modality of cancer treatment, the other three modalities being surgery, radiation, and chemotherapy. *See* Folkman, *Angiogenic Inhibitors: A Fourth Modality of Anticancer Therapy* 4 Community Oncology 296, 296-98 (2007) (cited in IDS of November 27, 2007). As of May 2007, nine angiogenic inhibitors were approved by the FDA and in more than 30 other countries to treat cancer. At least 50 other angiogenic inhibitors with varying degrees of antiangiogenic activity are in phase II and phase III clinical trials.

In the Final Office Action dated February 28, 2008, the Examiner dismisses the teachings of Kahn *et al.*, and Gerritsen *et al.*, because the models disclosed therein are *in vitro* models of angiogenesis and according to the Examiner do not provide a predictable expectation of success for inhibiting angiogenesis *in vivo*. However, if the art is such that a particular model is recognized as correlating to a specific condition, then the model should be accepted as correlating unless the Examiner has evidence that the model does not correlate. *In re Brana*, 51 F.3d at 1566; MPEP § 2164.02. The Examiner has failed to provide any evidence that the endothelial model for tube formation does not correlate to angiogenesis. The Examiner appears to admit that the tube formation assays are art recognized models of angiogenesis. *See* Final Office Action of February 20, 2008 at page 7.

The references provided by Applicants confirm the correlation of expression of stanniocalcin and angiogenesis described in Applicants' specification. In addition, the references further confirm the association of expression of stanniocalcin and tumor tissue as described in

Applicants' specification. The specification shows that stanniocalcin is expressed in ductal mammary adenocarcinoma, squamous cell carcinoma, chrondosarcoma, and renal cell carcinoma vasculature, but is not expressed in normal vessels. *See* the specification e.g. at page 145, line 32 to page 146, line 7 and Figures 28 and 29. Several of the post filing date references in the least confirm the correlation of expression of stanniocalcin in ductal mammary carcinoma and demonstrate similar results in other types of tumors. Therefore, one skilled in the art reading the specification would have a reasonable expectation that neutralizing or inhibiting antibodies to stanniocalcin would inhibit angiogenesis.

Applicants assert the evidence in the specification, as well as confirmatory evidence in the art shows that the specification enables the full scope of the claimed subject matter. Even if, in the unlikely event, that the Examiner is found to have met the initial burden for establishing that the claims are non enabled, Applicants submit they have provided evidence from others in the field that confirm the enablement of Applicants' claims. A number of different angiogenic models and different actual tumor tissue samples all consistently show a correlation between angiogenesis and the upregulation of expression of stanniocalcin in endothelial cells and tumor cells undergoing angiogenesis. One skilled in the art would expect based on Applicants' guidance and teachings in the specification and the knowledge in the art related to inhibition of angiogenesis with antibody antagonists that treatment of a tumor with an agent that inhibits or neutralizes stanniocalcin inhibits angiogenesis. For at least these reasons, Applicants request reversal of the rejection of all of the claims.

Claims 78 and 79

As claims 78 and 79 are dependent on independent claim 56, the arguments set forth above are relevant to the rejection of these claims and are incorporated herein.

As discussed above, the specification includes a working example showing upregulation of stanniocalcin precursor in an art recognized model for angiogenesis. *See* Example 19 in the specification at page 142 and Fig. 23. Stanniocalcin precursor expression was found to be dramatically enhanced under tube-forming conditions, demonstrating a strong correlation between expression of stanniocalcin and tube-formation. *See* the specification at page 25, lines 20-26 and Example 19 at page 142. Applicants also disclose that stanniocalcin is expressed in ductal mammary adenocarcinoma, squamous cell carcinoma, chrondosarcoma, and renal cell

carcinoma vasculature but not in normal vessels. *See* the specification at page 145, line 32 to page 146, line 7 and Figures 28 and 29. The combination of increased expression in endothelial cells in a model for angiogenesis, and increased expression in tumor tissue (such as ductal mammary adenocarcinoma, squamous cell carcinoma, chrondosarcoma, and renal cell carcinoma) provide a reasonable correlation of the relationship of upregulation of stanniocalcin with angiogenesis in ductal mammary adenocarcinoma, squamous cell carcinoma, chrondosarcoma, and renal cell carcinoma.

The teachings of the specification are also confirmed by other research in the field as discussed above. For example, McCudden *et al.* demonstrated that STC-1 and its receptor co-localized in breast cancer cells in 91% of cases. McCudden *et al.*, *Co-localization of Stanniocalcin-1 Ligand & Receptor in Human Breast Carcinomas* 213 Molecular Cellular Endocrinology 167 (2004) (cited in IDS of February 1, 2006 and April 11, 2007). STC-1 was shown to be localized in invasive and ductal carcinoma in situ using an antibody to STC-1. STC-1 mRNA was detected in breast cancer cells by *in situ* hybridization and correlated with primary tumor size, number of positive lymph nodes, and stage of the cancer cell. *See* Wascher *et al.*, *Stanniocalcin-1: A Novel Molecular Blood & Bone Marrow Marker for Human Breast Cancer* 9 Clinical Cancer Res. 1427 (2003) (cited in IDS of April 11, 2007). Stanniocalcin was also shown to be induced in human tumor cells, such as colon carcinoma, nasopharyngeal cancer, and ovarian cancer cultured under hypoxic conditions. *See* Yeung *et al.*, *Hypoxia-Inducible Factor-1 in Human Activation of Stanniocalcin-1 in Human Cancer Cells* 146 Endocrinology 4951 (2005) (cited in IDS of November 27, 2007).

These references confirm a correlation of upregulated expression of stanniocalcin in a breast carcinoma, renal cell carcinoma, squamous cell carcinoma, colon carcinoma, and prostate carcinoma. Therefore, one skilled in the art would have had a reasonable expectation that neutralizing or inhibiting antibodies to stanniocalcin would inhibit angiogenesis in breast carcinoma, renal cell carcinoma, squamous cell carcinoma, colon carcinoma, and prostate carcinoma. Applicants therefore request reversal of the rejection of claims 78-79.

CLAIMS APPENDIX

1-55. (canceled)

56. (previously presented) A method for inhibiting angiogenesis in a tumor comprising administering to the tumor an effective amount of an antibody or antigen binding fragment thereof that specifically binds and inhibits or neutralizes a polypeptide comprising an amino acid sequence of SEQ ID NO:76.

57-68. (canceled)

69. (previously presented) The method of claim 56, wherein the polypeptide is encoded by a polynucleotide comprising a nucleic acid sequence of SEQ ID NO:75.

70. (previously presented) The method of claim 56, wherein said antibody is a polyclonal antibody, a monoclonal antibody, an antibody fragment, a human antibody, a humanized antibody, a chimeric antibody, a bispecific antibody or a heteroconjugate antibody.

71. (previously presented) The method of claim 70, wherein said antibody is an antagonist or a neutralizing antibody.

72. (previously presented) The method of claim 56, wherein the antibody has polyepitopic specificity.

73. (previously presented) The method of claim 56, wherein the antibody is a human antibody, a chimeric antibody, or a humanized antibody.

74. (previously presented) The method of claim 56, wherein the antibody is an antibody fragment.

75. (previously presented) The method of claim 74, wherein the antibody fragment comprises a Fab fragment, a Fab' fragment, a $F(ab')_2$ fragment, or a Fv fragment.

76. (previously presented) The method of claim 56, wherein the antibody is a heteroconjugate antibody.

77. (previously presented) The method of claim 73, wherein the antibody is a humanized antibody.

78. (previously presented) The method of claim 56, wherein the tumor is selected from the group consisting of a breast carcinoma, renal cell carcinoma, squamous cell carcinoma, colon carcinoma, and prostate carcinoma.

79. (previously presented) The method of claim 78, wherein the tumor is a breast carcinoma.

80. (cancelled)

EVIDENCE APPENDIX

A. OFFICE ACTIONS AND AMENDMENTS/RESPONSE

1. Notice of Appeal filed May 20, 2008
2. Interview Summary mailed February 20, 2008
3. Final Office Action mailed February 20, 2008
4. Amendment filed November 27, 2008
5. Non-Final Office Action mailed June 28, 2007
6. RCE, Amendment and Response filed April 11, 2007
7. Advisory Action mailed March 1, 2007
8. Notice of Appeal, Amendment After Final filed February 13, 2007
9. Final Office Action mailed October 13, 2006
10. Amendment filed July 25, 2006
11. Non-Final Office Action mailed April 25, 2006
12. RCE, Amendment under 37 C.F.R. § 1.1116 filed February 1, 2006
13. Notice of Appeal filed November 8, 2005
14. Final Office Action mailed August 10, 2005
15. Amendment and Response filed May 24, 2005
16. Non-Final Office Action mailed November 24, 2004
17. RCE, Amendment and Response filed September 2, 2004
18. Non-Final Office Action mailed March 5, 2004
19. Petition Decision granted February 11, 2004
20. Petition for Revival, Response to Notice of Non-Responsive Election filed August 6, 2003

21. Notice of Abandonment mailed July 30, 2003

22. Notice of Non-Responsive Election mailed January 9, 2003

23. Restriction Response filed October 29, 2002

24. Amendment filed August 14, 2002

25. Restriction Requirement mailed August 5, 2002

26. Sequence Listing filed May 29, 2002

27. Notice to Comply mailed April 22, 2002

28. Sequence Listed filed February 22, 2002

29. Notice to Comply with Requirements Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures mailed January 23, 2002

30. Response to Notice to File Missing Parts August 24, 2001

31. Notice to File Missing Parts mailed February 22, 2001

32. Application filed October 31, 2000

B. REFERENCES RELIED UPON BY THE EXAMINER

1. U.S. Publication No. 2002/0042372 A1

2. U.S. Patent No. 5,773,876

3. Mook *et al.*, Biochim Biophys Acta, Vol. 1705:69-89, 2004, abstract

4. Dillman R.O., Annals of Internal Medicine, 111:592-603, 1989

5. Weiner L.N. Seminars in Oncology, 26 (4 Suppl 12): 41-50, August 1999

6. MSNBC News Services, "Mixed results on new cancer drug", November 9, 2000

7. Jain, Scientific American, July 1994

8. Gura, Science, v278, 1997, pp. 1041-1042

9. Alberts *et al.*, Molecular biology of the Cell, 3rd edition, 1994, page 465

10. Lewin, B., Genes VI, Oxford University Press, Inc. NY, Chapter 29, 1997

C. REFERENCES CITED BY APPELLANTS

1. Olsen *et al.*, *Human Stanniocalcin: a Possible Hormonal Regulator of Mineral Metabolism* 93 Proc. Nat'l Acad. Sci. USA 1792 (1996).

2. Davis *et al.*, *An α2β1 Integrin-Dependent Pinocytic Mechanism Involving Intracellular Vacuole Formation & Coalescence Regulates Capillary Lumen and Tube Formation in Three-Dimensional Collagen Matrix* 224 Experimental Cell Res.39 (1996).

3. Filvaroff *et al.*, *Stanniocalcin 1 Alters Muscle & Bone Structure & Function in Transgenic Mice* 143 *Endocrinology* 3681 (2002).

4. McCudden *et al.*, *Co-localization of Stanniocalcin-1 Ligand & Receptor in Human Breast Carcinomas* 213 Molecular Cellular Endocrinology 167 (2004).

5. Wascher *et al.*, *Stanniocalcin-1: A Novel Molecular Blood & Bone Marrow Marker for Human Breast Cancer* 9 Clinical Cancer Res. 1427 (2003).

6. Yeung *et al.*, *Hypoxia-Inducible Factor-1 in Human Activation of Stanniocalcin-1 in Human Cancer Cells* 146 Endocrinology 4951 (2005).

7. Kahn *et al.*, *Gene Expression Profiling in an* In Vitro *Model of Angiogenesis* 156 The Am. J. of Pathology 1887 (2000).

8. Gerritsen *et al.*, *In Silico Data Filtering to Identify New Angiogenesis Targets From a Large* In Vitro *Profiling Data Set* 10 Physiological Genomics 13 (2002).

9. Folkman, *Angiogenic Inhibitors: A Fourth Modality of Anticancer Therapy* 4 Community Oncology 296 (2007).

10. Eccles, *Monoclonal Antibodies Targeting Cancer: 'Magic Bullets' or Just the Trigger?* 3 *Breast Can. Res.*, 86 (2000).

11. Presta *et al.*, *Humanization of an Anti-Vascular Endothelial Growth Factor Monoclonal Antibody for the Therapy of Solid Tumors & Other Disorders* 57 Cancer Res. 4593 (1997).

13. Mook *et al.*, *The Role of Geltinases in Colorectal Cancer Progression & Mestasis* 1705 Biochemica Et Biophysica Acta 69 (2004).

14. Zlot *et al.*, *Stanniocalcin 1 is an Autocrine Modulator of Endothelial Angiogenic Responses to Hepatocyte Growth Factor* 278 Journal of Biological Chemistry 47654 (2003).

15. Press Release, Genentech, Inc. (Feb. 26, 2004).

D. CASES CITED IN THE BRIEF

1. *In re Wands*, 858 F.2d 731 (Fed. Cir. 1988).

2. *In re Angstadt*, 537 F.2d 498 (C.C.P.A. 1976).

3. *In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995).

4. *Cross v. Iizuka*, 753 F.2d 1040 (Fed. Cir. 1985).

5. *Atlas Powder Co. v. E.I. DuPont De Nemours & Co.*, 750 F.2d 1569 (Fed. Cir. 1984).

6. *In re Marzocchi*, 439 F.2d 220 (C.C.P.A. 1971).

7. *In re Wright*, 999 F.2d 1557 (Fed. Cir. 1993).

8. *Falko-Gunter vs. Inglis,* 448 F.3d 1357 (Fed. Cir. 2006).

9. *In re Cortright*, 165 F.3d 1353 (Fed. Cir. 1999).

RELATED PROCEEDINGS APPENDIX

None.

SUMMARY

It is earnestly requested that the Examiner's rejection be reversed for the reasons discussed herein, and that all of the pending claims be allowed. Applicants have provided evidence that both confirms the teachings of the specification and further establishes that one of skill in the art could practice the claims without undue experimentation. The Examiner has failed to provide a sufficient explanation or reasoning in any of the Office Actions as to why the claims lack enablement in view of Applicants' rebuttal evidence.

Please charge any additional fees or credit overpayment to Merchant & Gould Deposit Account No. 13-2725.

Respectfully submitted,

MERCHANT & GOULD P.C.
P.O. Box 2903
Minneapolis, Minnesota 55402-0903
(612) 332-5300

Date: August 19, 2008

Eric E. DeMaster
Reg. No. 55,107
EED:jrm

Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 1792–1796, March 1996
Biochemistry

Human stanniocalcin: A possible hormonal regulator of mineral metabolism

HENRIK S. OLSEN*†, MARIO A. CEPEDA*, QING-QING ZHANG*, CRAIG A. ROSEN*, BENITO L. VOZZOLO‡, AND GRAHAM F. WAGNER‡

*Human Genome Sciences, 9620 Medical Center Drive, Rockville, MD 20850-3338; and ‡Department of Physiology, Faculty of Medicine, University of Western Ontario, London ON, Canada N6A 5C1

Communicated by Henry G. Friesen, Medical Research Council of Canada, Ottawa, Canada, October 17, 1995

ABSTRACT **We have isolated a human cDNA clone encoding the mammalian homolog of stanniocalcin (STC), a calcium- and phosphate-regulating hormone that was first described in fishes where it functions in preventing hypercalcemia. STC has a unique amino acid sequence and, until now, has remained one of the few polypeptide hormones never described in higher vertebrates. Human STC (hSTC) was found to be 247 amino acids long and to share 73% amino acid sequence similarity with fish STC. Polyclonal antibodies to recombinant hSTC localized to a distinct cell type in the nephron tubule, suggesting kidney as a possible site of synthesis. Recombinant hSTC inhibited the gill transport of calcium when administered to fish and stimulated renal phosphate reabsorption in the rat. The evidence suggests that mammalian STC, like its piscine counterpart, is a regulator of mineral homeostasis.**

Stanniocalcin (STC) is a calcium-regulating hormone in bony fishes that has never been described in higher vertebrates, including mammals (1). The hormone is synthesized by the corpuscles of stannius (CS), endocrine glands that are associated with the kidneys of all fishes with a bony skeleton (2). The primary function of STC in fishes is the prevention of hypercalcemia and a rise in serum calcium levels is the primary stimulus for secretion (3). Upon release into the circulation, STC lowers calcium transport by the gills thereby reducing its rate of influx from the environment into the extracellular compartment (1). A second equally important action of STC is stimulation of phosphate reabsorption by renal proximal tubules (4). The consequence of this renal effect is increased levels of plasma phosphate, the latter of which combines with excess calcium and promotes its disposal into bone and scales. Because the CS have never been identified in higher vertebrates, it has long been assumed that STC was unique to fishes. However, recent evidence of STC immunoreactivity in human kidney and serum argues for a more widespread existence of the hormone (5).

By a process of random sequencing of human tissue cDNAs, we have isolated a lung-derived cDNA clone whose deduced protein sequence bears a strikingly high level of homology to salmon and eel STC (6, 7).§ Data indicating that human (h)STC inhibits calcium uptake in fish and phosphate excretion in rats suggest that hSTC is a hormonal regulator of mineral metabolism.

MATERIALS AND METHODS

cDNA Isolation and Analysis. *cDNA isolation.* The initial expressed sequence tag (EST) clones used in the study were discovered by scientists at The Institute for Genomic Research by using established EST methods (8, 9). These clones were part of a larger EST project (10). This clone was used for rescreening the same library and a full-length clone encoding hSTC was obtained.

Southern blot analysis. Ten-microgram aliquots of human genomic DNA were cut with *Bam*HI, *Eco*RI, and *Xba* I and then separated on an 0.8% agarose gel. After transfer to a nylon membrane, the blot was probed with ^{32}P-labeled hSTC cDNA and washed under high stringency.

Northern blot analysis. RNA was separated in a 0.8% agarose/formaldehyde gel in 1× Mops buffer (10× Mops = 0.2 M Mops/50 mM NaOAc/10 mM EDTA). After transfer to a nylon membrane, the blot was probed with ^{32}P-labeled hSTC cDNA and washed under high stringency, and the RNA expression pattern was analyzed by autoradiography.

Expression of hSTC in Bacteria. hSTC was cloned into the bacterial expression (pQE) vector and a histidine-tagged protein was purified by ion-affinity column chromotography according to the manufacturer (Quiagen, Chatsworth, CA).

Briefly, the bacterial culture harboring the hSTC expression plasmid was grown to an OD_{600} of 0.4, induced with 1 mM isopropyl β-D-thiogalactoside, and grown for an additional 4 h. After induction, the cell pellet was dissolved in 6 M guanidine hydrochloride (pH 8.0) and applied to the ion-affinity column. After extensive washing, the protein was renatured by applying a gradient of urea (4 M to 0 M) in 150 mM NaCl/10% (vol/vol) glycerol. Protein was eluded in 250 mM imidazole/150 mM NaCl/25 mM Tris/10% glycerol and dialyzed.

Fish Bioassay. For testing the effects of hSTC in fishes, an established fish STC bioassay was employed essentially as described (1). This bioassay monitors the inhibitory effects of STC on gill calcium transport. Goldfish (1.2 ± 0.1 g per 10 fish per group) were given two intraperitoneal injections, 1 h apart, of recombinant hSTC, purified salmon STC, or saline, and then placed in tanks of water containing ^{45}Ca (100,000 dpm/ml) for a 3-h period. The fish were then sacrificed and ashed overnight in a muffle furnace, and the isotope content of the resulting ash was determined by scintillation counting. The rate of gill calcium transport in each fish was determined on the basis of body isotope content and water-specific activity and was expressed as μmol of Ca^{2+} per kg (body weight) per h. Individual treatment groups were considered significantly different than solvent-injected controls if $P < 0.05$ (ANOVA and Dunnet's test).

Immunocytochemistry. Antibodies were prepared in rabbits after three monthly immunizations, each of 200 μg of bacterially expressed recombinant hSTC dissolved in Freund's adjuvant/saline, 1:1 (vol/vol). The development of antibody titer was monitored by ELISA and the highest titer was obtained after the third immunization. Western blot analysis of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: STC, stanniocalcin; h, human; ICC, immunocytochemistry; CS, corpuscles of stannius; STCir, STC immunoreactive.

†To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U46768).

human kidney extracts and recombinant hSTC yielded two closely spaced bands in both instances. Fresh human kidney biopsies were obtained from Surgical Pathology, University Hospital, London, with patient consent, and immediately fixed overnight in PBS containing 4% (wt/vol) paraformaldehyde (pH 7.4). Tissues were dehydrated, embedded in paraffin, and 4-μm serial sections were mounted on coated slides. For immunocytochemistry (ICC), tissue sections were dewaxed, rehydrated, and equilibrated in ICC diluent buffer (0.025 M Tris, pH 7.5/0.15 M NaCl). Sections were then treated for 10 min with undiluted normal goat serum to reduce background staining prior to an overnight incubation with a 1:1000 dilution of hSTC antiserum. After an extensive wash in ICC buffer, the sections were incubated for 1 h in peroxidase-coupled goat anti-rabbit IgG. The sites of antibody binding were visualized with 0.025% diaminobenzidene prepared in ICC buffer containing 0.01% H_2O_2. After development, the sections were counterstained in hematoxylin, dehydrated, and mounted. Control procedures included the application of normal rabbit serum or hSTC antiserum preabsorbed with hSTC in lieu of antiserum alone.

Rat Bioassay. Recombinant hSTC was tested for effects in rats by using standard methods for assessment of renal function. Male Wistar rats (300 g) were anesthetized with Inactin (100 mg/kg) and ketamine (10 mg/kg), and a tracheostomy was performed to facilitate breathing. Body temperature was maintained at 37 ± 0.5°C with a heating pad. A right jugular vein cannula was used for the infusion of saline and test substances. A right femoral artery cannula was used for monitoring mean arterial pressure and for blood sampling. Both ureters were cannulated near the renal pelvis for urine collection. The animals were infused with saline for 1 h after surgery prior to commencing an experiment. To start an experiment, urine was collected over the first 20-min interval, C1, and a blood sample was taken at the midpoint of urine collection. Recombinant hSTC or solvent was given as a 500-μl bolus 15 min into the second collection period, C2. Urine and blood were then collected over the next 80 min, C3 through C6. At the end of the experiment, the animals were sacrificed and the kidneys were removed, decapsulated, and weighed. Urine volume was calculated gravimetrically and expressed as ml per min per g (kidney weight). Mean arterial pressure was determined for each clearance period by averaging the readings over each clearance period. Plasma and urinary concentrations of Na^+ and K^+ were determined by flame photometry, whereas calcium (11) and inorganic phosphate (12) were determined colorimetrically. On the basis of these analyses, electrolyte excretion rates in clearance periods C3 through C6 were expressed as changes from C1 levels. The data were analyzed by repeated measures ANOVA and groups were considered significantly different if $P < 0.05$.

RESULTS AND DISCUSSION

A partial cDNA clone with sequence similarity to salmon STC (6) was isolated by random sequencing of an early-stage human fetal lung cDNA library. This clone was used for rescreening the same library and a full-length clone encoding hSTC was obtained. The human cDNA encodes a protein that is 247 amino acids long (Fig. 1) and the level of sequence similarity when compared to salmon STC (6) was 92% over the first 204 amino acids, of which 118 residues were identical (Fig. 2). The last 43 residues on the C-terminal end of hSTC were completely divergent. However, this has also proven to be the case among fish species (6) and suggests that the C-terminal end is not critical for biological activity. Several features are conserved between fish STC and hSTC. For instance, the 11 half cystines in the mature protein core that participate in inter- and intrachain disulfide bonding occupy the same positions in salmon STC (6) and hSTC. This implies that the human protein

```
MLQNSAVLLVLVISASATHEAEQNDSVSPRKSRV

AAQNSAEVVRCLNSALQVGCGAFACLENSTCDTD

GMYDICKSFLYSAAKFDTQGKAFVKESLKCIANG

VTSKVFLAIRRCSTFQRMIAEVQEECYSKLNVCS

IAKRNPEAITEVVQLPNHFSNRYYNRLVRSLLEC

DEDTVSTIRDSLMEKIGPNMASLFHILQTDHCAQ

THPRADFNRRRTNEPQKLKVLLRNLRGEEDSPSH

IKRTSHESA
```

FIG. 1. Deduced amino acid sequence of hSTC. A partial cDNA clone with sequence similarity to salmon STC (6) was isolated by random sequencing of an early-stage human fetal lung cDNA library. Commencing with an initiator methionine and terminating with a stop codon, the human cDNA encodes a protein that is 247 amino acid residues long. The glycosylation consensus sequence is underlined.

is a homodimer in the native state as in the case of salmon. Salmon STC contains an additional half cystine in the hydrophobic leader sequence, however, that is not conserved in the human protein (6). The glycosylation consensus sequence Asn-Ser-Thr has also been conserved (6, 7, 13), indicating that hSTC is glycosylated like its piscine counterpart. Indeed, baculovirus-expressed hSTC binds readily to the plant lectin, concanavalin A, a feature that has been exploited in purifying the recombinant glycosylated protein (14, 15). In salmon, pro-STC is cleaved between Arg-33 and Phe-34 to yield a 223-residue mature protein core with phenylalanine on the N terminus (6). We have not yet established the correct cleavage site for mature human STC.

To assess the biological effects of hSTC, the bacterially expressed protein was injected into fish and rodents. For these studies, the entire open reading frame of the cDNA clone was placed in a bacterial expression vector containing a histidine tag sequence and the expressed protein was purified on a metal-ion affinity column. The ability of hSTC to mimic the fish hormone was verified in an established bioassay that measures the inhibitory effects of STC on gill calcium transport in fishes (1). In response to intraperitoneal injections of both hSTC (10 mg/kg) and salmon STC (1 mg/kg), gill calcium transport in the goldfish was significantly reduced in comparison to saline-injected controls (Fig. 3). This indicated that hSTC was indeed bioactive and capable of substituting for the fish hormone, presumably by binding to the fish STC receptor.

Recent findings suggest that the purified fish hormone has limited effects, if any, on calcium metabolism in mammals, particularly the parameters affected by parathyroid hormone such as serum calcium, bone resorption, and urinary cAMP (16). Salmon STC does, however, promote phosphate reabsorption by the fish kidney (4), which is an indirect mechanism for the lowering of plasma calcium levels. With this in mind, the effects of recombinant hSTC on phosphate reabsorption were assessed in anesthetized male rats by using standard clearance procedures for estimating renal function (17). In response to a single bolus injection [5 nmol of hSTC per kg (body weight)], phosphate excretion was significantly decreased in the rat (Fig. 4) with no concomitant effects on other plasma and urinary electrolytes or renal function. A similar effect was seen with baculovirus-expressed hSTC. Hence, recombinant hSTC also proved capable of mimicking an established effect of STC in fishes when tested in a mammalian model system.

```
                                  10        20        30
Human                MLQNSAVLLVLVISASATHEAEQNDSVSPRKSRVAAQN
                               : ||:::::|| ::::::: |||::| ::::
Salmon               MLAKFGLCAVFLVLGTAATFDTDPEEA-SPRRARFSSNS

 40        50        60        70        80        90
SAEVVRCLNSALQVGCGAFACLENSTCDTDGMYDICKSFLYSAAKFDTQGKAFVKESLKC
:::|:||||:||:||||:|||||||||||||||:|||: |:::||:|:||||:|||||||:|
PSDVARCLNGALAVGCGTFACLENSTCDTDGMHDICQLFFHTAATFNTQGKTFVKESLRC
  7
100       110       120       130       140       150
IANGVTSKVFLAIRRCSTFQRMIAEVQEECYSKLNVCSIAKRNPEAITEVVQLPNHFSNR
|||||||||| :||||::||||||:||||||||:|::|::|::|||||:||||:|:||:||
IANGVTSKVFQTIRRCGVFQRMISEVQEECYSRLDICGVARSNPEAIGEVVQVPAHFPNR

160       170       180       190       200       210
YYNRLVRSLLECDEDTVSTIRDSLMEKIGPNMASLFHILQTDHCAQTHPRADFNRRRTNE
||: |::|||:|||:||:::|::|::::||:|::||::||::||:|
YYSTLLQSLLACDEETVAVVRAGLVARLGPDMETLFQLLQNKHCPQGSNQGPNSAPAGWR

220       230       240
PQKLKVLLRNLRGEEDSPSHIKRTSHESA

WPMGSPPSFKIQPSMRGRDPTHLFARKRSVEALERVME
```

FIG. 2. Amino acid sequence comparison of hSTC and salmon STC. A solid line between species denotes identity and double dots denote similarity. Note the high level of identity in the core of the molecule. The level of sequence similarity when compared to salmon STC (6) was 92% over the first 204 amino acids, of which 118 residues were identical. The underlined sequence denotes the glycosylation consensus sequence. The last 43 residues on the C-terminal end of hSTC were completely divergent from the salmon, as is the case between the different fish STCs (6, 7). Sequence alignment was done with the University of Wisconsin Genetics Computer Group TFASTA program (182).

The hSTC gene is present as a single copy according to Southern blot analysis. To identify its chromosomal locus, a genomic clone was isolated and used in fluorescent *in situ* hybridization as described (18). The analysis of chromosomal spreads indicated that the hSTC gene was localized to band 8p21. When the tissue distribution of STC gene expression was examined by Northern blot analysis, low levels of expression were detected in several tissues including kidney, bone marrow, and thymic stromal cells (Fig. 5). ICC (19) was employed to identify cellular sources of the protein in human kidney by using a polyclonal antiserum to recombinant hSTC. In the kidney, no immunoreactivity was detected in the glomeruli, vascular elements, or hematopoietic tissue surrounding the nephrons; STC-immunoreactive (STCir) cells were confined to the nephron, specifically in distal convoluted tubule and the collecting tubule. Some STCir cells in the collecting tubule were phenotypically unique due to their large size and ten-

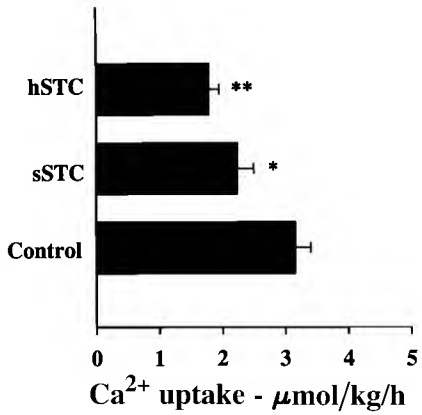


FIG. 3. hSTC inhibits gill calcium transport in fish. hSTC and salmon STC (sSTC) were tested for effects on gill calcium transport as described (1). Goldfish (1.2 ± 0.1 g; 10 fish per group) were given intraperitoneal injections of hSTC (10 mg/kg), salmon STC (1 mg/kg), or saline and placed in tanks of ^{45}Ca-containing water (50,000 dpm/ml) for 3 h. The fish were then sacrificed and individually ashed overnight at 600°C, and the isotope content of the ash was determined by scintillation counting. Based on the body weight of the fish and the specific activity of the water, gill calcium transport in each fish was expressed as μmol of Ca^{2+} per kg (body weight) per h. Both hSTC (**, $P < 0.01$) and salmon STC (*, $P < 0.05$) had statistically significant inhibitory effects on gill calcium transport (two-tailed ANOVA and Dunnet's test).

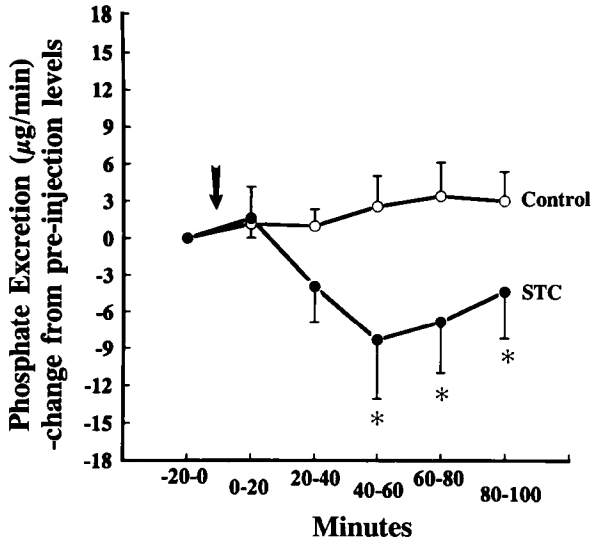


FIG. 4. hSTC inhibits phosphate excretion in the rat. Rats (250 ± 10 g; 5 rats per group) were maintained under Inactin anesthesia with catheters in one jugular vein, one carotid artery, and both ureters and were continuously infused with inulin and *p*-aminohippuric acid for measurement of glomerular filtration and renal blood flow, respectively, as described (15). Bolus injections of hSTC (5 nmol/kg) or saline were given via the jugular catheter after the first urine collection period (arrow) and renal function, plasma and urinary electrolytes (Na^+, K^+, Ca^{2+}, Mg^{2+}, and PO_4), and blood pressure were monitored throughout. Phosphate excretion was maximally inhibited by hSTC after 60 min and remained significantly different thereafter ($P < 0.01$; repeated measures ANOVA). STC had no effect on any other parameters.

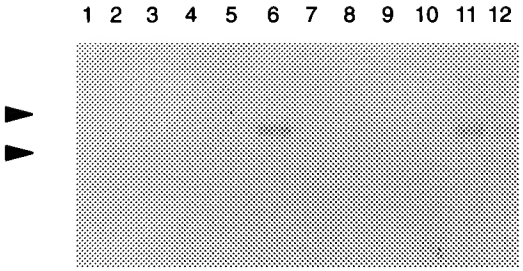


FIG. 5. Northern blot analysis of hSTC mRNA expression. The hSTC cDNA was radiolabeled and hybridized to 10 μg of total RNA unless otherwise indicated. A 1-week exposure of the blot is shown. Lanes: 1, stomach; 2, thymus; 3, spleen; 4, peripheral T cells; 5, leukocytes; 6, kidney; 7, lung; 8, liver; 9, heart; 10, pancreas; 11, thymic stromal cells (1.5 μg); 12, bone marrow [1 μg of poly(A) RNA]. The arrowheads at left denote the positions of 18S (bottom) and 28S (top) rRNAs.

dency to have multiple nuclei (Fig. 6*A*), suggesting that they were functionally different than surrounding cells. Antibody staining of STCir cells was abolished when the primary antiserum was preabsorbed with hSTC (Fig. 6*B*). In fishes, the CS glands are derived from the kidneys. Individual STC cells bud off from the nephron tubules during embryogenesis, coalesce to form CS glands, and then migrate to the kidney surface prior to hatching (20). In the most primitive bony fishes, however, the glands remain deep within the kidneys associated with individual nephrons (21). Therefore, the presence of STCir cells in the mammalian nephron is entirely consistent when viewed from an evolutionary perspective. The ICC results obtained in the present study with hSTC antiserum have confirmed and extended earlier ICC findings in human kidney using antibodies to the fish hormone. In the case of both antisera, staining was confined to the nephron tubule, as no staining was observed in glomeruli, proximal tubules, vascular elements, or hematopoietic tissue. However, whereas both antiserum stained distal tubule cells, the hSTC antiserum also stained cells in the thick ascending limb and collecting tubules. As our fish antiserum is highly crossreactive with recombinant hSTC, the different ICC staining patterns obtained with the two antiserum may be indicative of there being different STC cell types.

The hormonal regulation of mineral homeostasis in mammals is a complex process involving parathyroid hormone (22, 23), calcitonin (24), and the active metabolite of vitamin D (25). Parathyroid hormone and vitamin D counteract hypocalcemia by stimulating bone resorption, as well as intestinal and renal calcium transport, and parathyroid hormone also enhances renal phosphate excretion. Calcitonin, on the other hand, counteracts hypercalcemia by inhibiting osteoclastic bone resorption, although its importance as a minute-to-minute regulator of calcium homeostasis remains controversial (26). The complexity of mineral homeostasis is reflected in the numerous diseases associated with the impaired regulation of calcium and phosphate, which impact on renal function, and the vascular, neuronal, and muscular systems, in addition to bone mineralization. Recent findings involving tumor-induced osteomalacia in humans (27) and murine models of X chromosome-linked hypophosphatemia (28) imply there is room for additional humoral regulators of mineral homeostasis in mammals and STC warrants consideration as a candidate. Given the structural similarities between hSTC and fish STC and their comparable effects on renal function, STC may have the same role in mammals and fishes: preventing hypercalcemia, in part, through its stimulatory effects on phosphate reabsorption. The remarkable evolutionary conservation between fish and mammalian STC suggests an important role for this hormone in higher vertebrates.

We thank Human Genome Sciences and The Institute for Genomic Research sequencing facilities for cDNA sequencing; K. Carter and B. Shell for the fluorescence *in situ* hybridization mapping. We also thank Dr. R. L. Kline (Department of Physiology, University of Western Ontario) for assistance with the rat studies. Grant and Scholarship

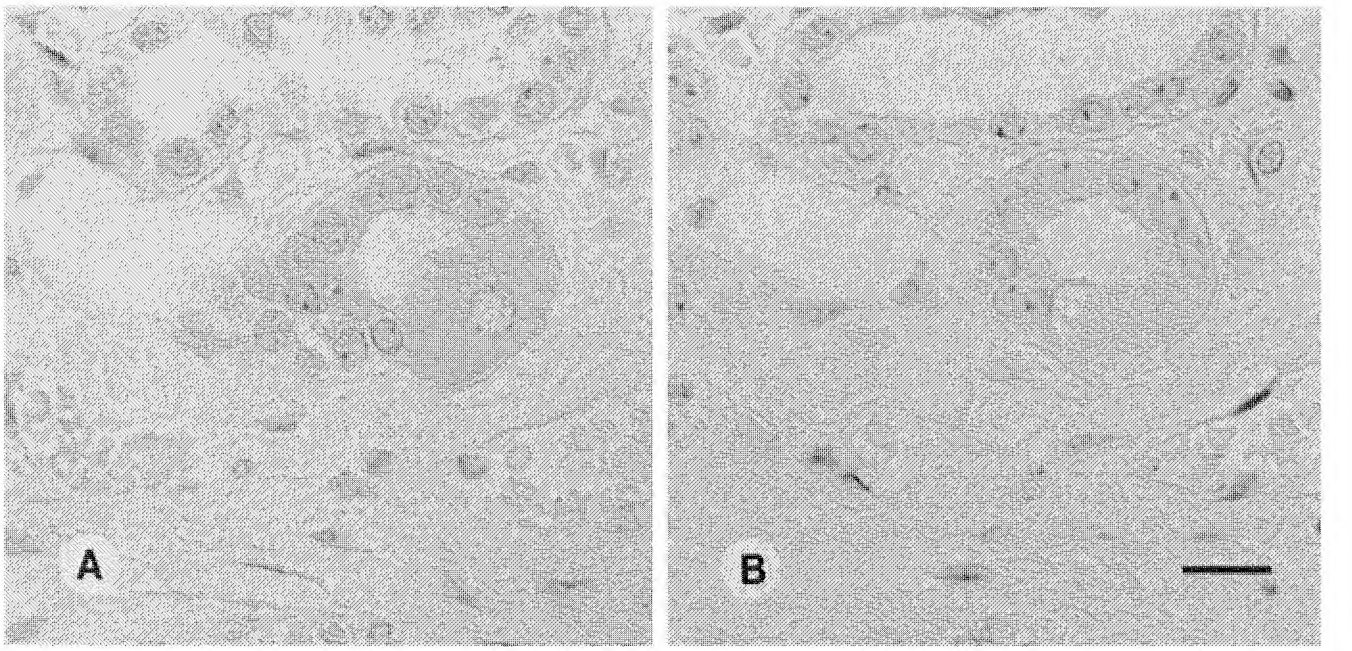


FIG. 6. STCir cells are present in human kidney nephron tubule. ICC was performed as described (19). Fresh biopsies of human kidney were fixed overnight in phosphate-buffered 4% (wt/vol) paraformaldehyde (pH 7.2), dehydrated, and embedded in paraffin. Dewaxed 5-μm serial sections were incubated overnight with a 1:1000 dilution of rabbit anti-hSTC serum in Tris-buffered saline (pH 7.5) (*A*) or the same antiserum dilution preabsorbed with recombinant hSTC (*B*). The slides were then washed for three 10-min periods in Tris-buffered saline, incubated for 30 min with peroxidase-coupled goat anti-rabbit IgG, washed as before, and developed in 0.025% diaminobenzidene containing 0.01% hydrogen peroxide. (*A*) Large multinucleated cell in the collecting tubule portion of the nephron in outer medullary kidney stained by the antiserum. (*B*) Adjacent section treated with preabsorbed antiserum and showing no specific staining. (Bar = 15 μm.)

support from the Medical Research Council of Canada awarded to G.F.W. is also gratefully acknowledged.

Note Added in Proof. While this manuscript was in review, a similar sequence was reported by Chang *et al.* (29).

1. Wagner, G. F., Hampong, M., Park, C. M. & Copp, D. H. (1986) *Gen. Comp. Endocrinol.* **63,** 481–491.
2. Stannius, H. (1839) *Arch. Anat. Physiol.* **6,** 97–101.
3. Wagner, G. F., Milliken, C., Friesen, H. G. & Copp, D. H. (1991) *Mol. Cell. Endocrinol.* **79,** 129–138.
4. Lu, M., Wagner, G. F. & Renfro, J. L. (1994) *Am. J. Physiol.* **36,** R1356–R1362.
5. Wagner, G. F., Guiraudon, C. C., Milliken, C. & Copp, D. H. (1995) *Proc Natl Acad Sci.* **92,** 1871–1875.
6. Wagner, G. F., Dimattia, G. E., Davie, J. R., Copp, D. H. & Friesen, H. G. (1992) *Mol. Cell. Endocrinol.* **90,** 7–15.
7. Butkus, H., Roche, P. J., Fernley, R. T., Haralambidis, J., Penschow, J. D., Ryan, G. B., Trahair, J. F., Tregear, G. W. & Coughlin, J. P. (1987) *Mol. Cell. Endocrinol.* **54,** 123–134.
8. Adams, M. D., Kelley, J. M., Gocayne, J. D., Dubnick, M., Polymeropoulos, M. H., Xiao, H., Merril, C. R., Wu, A., Olde, B. & Moreno, R. F. (1991) *Science* **252,** 1651–1656.
9. Adams, M. D., Dubnick, M., Kerlavage, A. R., Moreno, R., Kelley, J. M. & et al., (1992) *Nature (London)* **355,** 632–634.
10. Adams, M. D., Kerlavage, A. R., Fleischmann, R. D. & Fuldner, R. A. (1995) *Nature (London)* **377,** Suppl., 3–174.
11. Baginski, E. S., Marie, S. S., Clark, W. L. & Zak, B. (1973) *Clin. Chem. Acta* **46,** 46–54.
12. Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28,** 1756–1758.
13. Wagner, G. F. (1994) in *Fish Physiology*, eds. Sherwood, N. & Hew, C. (Academic, New York), Vol. 13, pp. 273–306.
14. Summers, M. D. & Smith, G. E. (1987) *Tex. Agric. Exp. Stn. Bull.* **1555** (abstr.).
15. Luckow, V. A. & Summers, M. D. (1989) *Virology* **170,** 31–39.
16. Stern, P. H., Shanker, G. L., Fargher, R. C., Copp, D. H., Milliken, C. E., Sato, K., Goltzman, D. & Herrmann-Erlee, M. P. M. (1991) *J. Bone Miner. Res.* **11,** 1153–1159.
17. McLennan, G. P., Kline, R. L. & Mercer, P. F. (1991) *Hypertension* **17,** 54–62.
18. Papadopoulos, N., Nicolaides, N. C., Wei, Y.-F., Ruben, S. M., Carter, K. C., Rosen, C. A., Haseltine, W. A., Fleischmann, R. D., Fraser, C. M., Adams, M. D., Venter, J. C., Hamilton, S. R., Petersen, G. M., Watson, P., Lynch, H. T., Peltomaki, P., Mecklin, J.-P., de la Chapelle, A., Kinzler, K. W. & Vogelstein, B. (1994) *Science* **263,** 1625–1629.
19. Wagner, G. F., Copp, D. H. & Friesen, H. G. (1988) *Endocrinology* **122,** 2064–2070.
20. Garrett, F. D. (1942) *J. Morphol.* **70,** 41–67.
21. Youson, J. H. & Butler, D. G. (1976) *Acta Zool. (Stockholm)* **57,** 217–238.
22. Brown, E. M., LeBoff, M. S., Oetting, M., Possilico, J. T. & Chen, C. (1987) *Res. Prog. Horm. Res.* **43,** 337–382.
23. Aurbach, G. D. (1988) *Calcium in Human Biology* (Springer, London), p. 43.
24. Breimer, L. H., MacIntyre, I. & Zaidi, M. (1988) *Biochem. J.* **255,** 377–390.
25. DeLuca, H. F., Krisinger, J. & Darwish, H. (1990) *Kidney Int.* **38,** S2–S8.
26. Munson, P. L. & Hirsch, P. F. (1992) *J. Bone Miner. Res.* **16,** 162–165.
27. Cai, Q., Hodgson, S. F., Kao, P. C., Lennon, V. A., Klee, G. C., Zinsmiester, A. R. & Kumar, R. (1994) *N. Engl. J. Med.* **330,** 1645–1649.
28. Meyer, R. A., Meyer, M. H. & Gray, R. W. (1989) *J. Bone Miner. Res.* **4,** 493–500.
29. Chang, A. C., Janosi, J., Hulsbeek, M., de Jong, D., Jeffrey, K. J., Noble, J. R. & Reddel, R. R. (1995) *Mol. Cell. Endocrinol.* **112,** 241–247.

0013-7227/02/$15.00/0
Printed in U.S.A.

Endocrinology 143(9):3681–3690

doi: 10.1210/en.2001-211424

Stanniocalcin 1 Alters Muscle and Bone Structure and Function in Transgenic Mice

ELLEN H. FILVAROFF, SUSAN GUILLET, CONSTANCE ZLOT, MIN BAO, GLADYS INGLE, HOPE STEINMETZ, JOHN HOEFFEL, STUART BUNTING, JED ROSS, RICHARD A. D. CARANO, LYN POWELL-BRAXTON, GRAHAM F. WAGNER, RENEE ECKERT, MARY E. GERRITSEN, AND DOROTHY M. FRENCH

Departments of Molecular Oncology (E.H.F., M.B.), Pathology (S.G., D.M.F.), Physiology (C.Z., G.I., H.S., J.H., S.B., J.R., R.A.D.C., L.P.-B., M.E.G.), and Safety Assessment (R.E.), Genentech Inc., South San Francisco, California 94080; and Department of Physiology (G.F.W.), Faculty of Medicine and Dentistry, The University of Western Ontario, London, Ontario N6A 5C1, Canada

Fish stanniocalcin (STC) inhibits uptake of calcium and stimulates phosphate reabsorption. To determine the role of the highly homologous mammalian protein, STC-1, we created and characterized transgenic mice that express STC-1 under control of a muscle-specific promoter. STC-1 transgenic mice were smaller than wild-type littermates and had normal growth plate cartilage morphology but increased cartilage matrix synthesis. In STC-1 mice, the rate of bone formation, but not bone mineralization, was decreased. Increased cortical bone thickness and changes in trabeculae number, density, and thickness in STC-1 mice indicated a concomitant suppression of osteoclast activity, which was supported by microcomputed tomography analyses and histochemistry. Skeletal muscles were disproportionately small and showed altered function and response to injury in STC-1 mice. Electron microscopy indicated that muscle mitochondria were dramatically enlarged in STC-1 mice. These changes in STC-1 mice could not be explained by deficits in blood vessel formation, as vascularity in organs and skeletal tissues was increased as was induction of vascularity in response to femoral artery ligation. Our results indicate that STC-1 can affect calcium homeostasis, bone and muscle mass and structure, and angiogenesis through effects on osteoblasts, osteoclasts, myoblasts/myocytes, and endothelial cells. (*Endocrinology* 143: 3681–3690, 2002)

STANNIOCALCIN (STC), A SECRETED protein, was first discovered in fish, where it regulates calcium and phosphate homeostasis (1–3). More specifically, STC in fish inhibits uptake of calcium through the gills and intestines and stimulates phosphate reabsorption (1–3). The mammalian homolog of fish STC, human STC-1, shares approximately 80% amino acid similarity with fish STC (4, 5). As in fish, mammalian STC-1 controls phosphate transport across epithelia in the gut and kidney (6, 7). Mammalian STC-1 is expressed in many tissues (4, 5, 8). However, very little is known about mammalian STC-1 activity, and hypotheses about its function have been based largely on studies of the pattern of STC-1 expression during growth and development.

Recent data suggest that STC-1 plays a role in the nervous system. STC-1 is constitutively expressed in terminally differentiated neurons in the central nervous system (9), and culture of a human neural-crest-derived cell line, Paju, in hypercalcemic conditions induces STC-1 expression (10). STC-1 is up-regulated in neurons around infarcts in human and rat brains (10) and in hypoxic human glioblastoma cells (11). Because calcium mobilization and influx may mediate, at least in part, toxicity after ischemia, STC-1 may protect cells from hypoxic/ischemic insult (10). In fact, STC-1 can protect Paju cells after ischemic challenge and increase cellular uptake of inorganic phosphate (10). Expression of STC in the epithelium of the choroid plexus suggests a role for STC in the regulation of calcium and phosphate levels in cerebrospinal fluid and blood (12).

STC-1 has also been implicated in endothelial activation and angiogenesis. STC-1 is expressed at high levels in highly vascularized tissues such as the heart, kidney, and lung as well as the ovary, and it is up-regulated during gestation and lactation (4, 5, 13, 14). Consistent with its high, ubiquitous expression, STC-1 may also play a role in mesenchymal-epithelial interactions (15). STC-1 mRNA is highly up-regulated in endothelial cells undergoing differentiation into tube-like structures, and STC-1 mRNA is localized to blood vessels in tumors (16). In fact, STC-1 is expressed in 95% of cancer cell lines tested and in many types of tumors (17). Furthermore, STC-1 mRNA levels are higher in hepatocellular carcinoma and colorectal tumors than in normal tissues (17).

Based on its presence in chondrocytes and muscle cells, a role for STC-1 protein in chondrogenic and myogenic differentiation has been suggested (18). More specifically, STC-1 protein is present in developing bones and muscles of the mouse fetus. During bone development, STC-1 is found in chondroprogenitors, chondrocytes and osteoblasts (18–20). Just before ossification during intramembranous bone formation, STC-1 is present in the mesenchyme. During muscle development, STC-1 is found in heart myocardiocytes and at

Abbreviations: BV/TV, Bone volume per total volume; μCT, microcomputed tomography; DA, degree of anistropy; STC, stanniocalcin hSTC-1, human STC-1; mAb, monoclonal antibody; MIL, mean intercept length; PECAM, platelet-endothelial cell adhesion molecule; TRAP, tartrate-resistant acid phosphatase; VOI, volume of interest; WT, wild-type.

all stages of skeletal myoblast differentiation into myotubes (18). Recent data suggest that, as in other tissues (15), STC-1 signals between adjacent cell types in the skeleton (20).

In spite of a number of correlative studies, the exact function of STC-1 remains unknown. To explore the function of STC-1 in mammals, transgenic mice expressing human STC-1 under control of a muscle-specific promoter were generated. These transgenic mice expressed high levels of STC-1 in skeletal muscle and were significantly smaller than their wild-type (WT) littermates. Decreases in body weight were significant starting as early as 5 d following birth, and body weight differences were maintained throughout the life of the animals. Detailed analyses revealed substantial changes in the structure and function of the musculoskeletal system. Given the high, ubiquitous expression of STC-1 (4, 5, 8), our results provide insight into possible mechanism(s) of action of mammalian STC-1.

Materials and Methods

Generation of STC-1 transgenic mice

All protocols were approved by the Institutional Animal Care and Use Committee. The gene for human STC-1 (hSTC-1) was cloned downstream of the rat myosin light chain 2 promoter sequence (21) and was followed by the splice donor/acceptor sites (between exons 4 and 5) of the human GH gene (22). Transgenic mice were made using this construct as per standard procedures (23). Pups were genotyped at 9 d of age by PCR of mouse tail DNA (QIAGEN, Santa Clarita, CA). Transgene expression was assessed in tissue (muscle, kidney, liver, intestine) biopsies taken at 4 wk of age by RT-PCR or Northern blot analysis (QIAGEN) of mRNA using hSTC-1 primers for exons 1–4.

All analyses were performed on adult mice except where noted in the text. Statistical analysis of differences between transgenic (STC-1) and WT mice was determined by a Student's t test with a value of $P < 0.05$ considered to be statistically significant and $P < 0.01$ as highly significant.

Whole animal physiology

All animals were kept on a 12-h light cycle and fed standard rodent chow. Mice were weaned and single housed in microisolater cages at 3 wk of age. Six founders (three males, three females) were examined. Once the phenotype was determined to be consistent among founders, two founders with highest human STC-1 expression, as determined by Taqman analysis, were bred to establish lines. Data presented are from offspring from one founder line unless otherwise indicated. Body weights, evaluated every other day from 5–42 d of age for male and female mice (n = 10 for each group of transgenic or WT mice) were used to establish comprehensive growth curves. Organs—brain, heart, left kidney, right kidney, liver, lung, spleen, and thymus—were weighed at 14 wk of age.

Clinical chemistry was performed on serum samples taken at 50 d of age (n = 10 mice of each genotype). Pooled sera from male and female mice (n = 5/group) were sent to AniLytics, Inc. (Gaithersburg, MD) for analysis by immunoassay of the following hormones: calcitonin, thyroid hormone (T_4), and GH. At 10 wk of age, founders were housed in metabolic chambers to assess food intake and oxygen consumption followed by a glucose tolerance test.

Whole body radiographs using a Faxitron x-ray (MX20, IDL software) were taken at 25-kV/120-sec exposures using Kodak X-Omat TL film (Eastman Kodak Co., Rochester, NY). Film was developed in a Kodak processor and scanned into Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA). Bone (femur and tibia) lengths were determined by manual measurements from planar x-rays.

Oxygen consumption was measured in a Columbus Instruments Oxymax open circuit calorimeter (Columbus, OH).

Histology

The following tissues were collected and fixed overnight in 10% neutral buffered formalin: skeletal muscle, ovary, testis, skin/mammary gland, lymph node, liver, intestines, pancreas, lungs, spleen, kidney, heart, aorta, thymus, urinary bladder, adrenal gland, pituitary gland, brain, and eye. After fixation, tissues were embedded in paraffin, sectioned at 5 μm, and stained with hemotoxylin and eosin or alcian blue (femurs). Femurs from a separate group of transgenic and WT mice were fixed in 70% ethanol, embedded in methyl-methacrylate, and stained using the Von Kossa method.

For osteoclast detection using histochemical staining for tartrate-resistant acid phosphatase (TRAP) activity, skulls were fixed in 2% paraformaldehyde overnight, equilibrated in methanol, and then TRAP stained using the leukocyte acid phosphatase kit (Sigma, St. Louis, MO).

Histomorphometry

For kinetic analyses, 6-wk-old STC-1 transgenic and WT mice were given ip injections of 2 mg/kg calcein, followed by similar injections 5 d later. Femur and tibia, harvested 2 d after the second injection, were dissected and fixed in 70% ethanol. Bones were embedded in the same orientation in methyl-methacrylate, sectioned at 10 μm, and viewed under UV light. Mineral apposition rate was determined following the recommended nomenclature (24).

Tissue processing for EM

Tissues were fixed in 1/2 Karnovsky's (2% paraformaldehyde; 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) and then washed in 0.1 M sodium cacodylate (pH 7.4), 2 × 15 min before being postfixed in 1% aqueous osmium tetroxide for 2 h at room temperature. Following washing in double-distilled H_2O for 2 × 15 min, the samples were dehydrated through an EtOH series: 50%, 70%, 90%, for 15 min each, and then 100% 2 × 15 min, followed by propylene oxide for 2 × 15 min. Samples were infiltrated with Eponate 12 (Ted Pella, Redding, CA) first with 1:1 propylene oxide:Eponate 12 overnight, followed by 100% Eponate 12 for 8 h, and then transferred to fresh resin and polymerized in a 60 C oven overnight and ultrathin (80 nm) sections were cut. Sections were stained with uranyl acetate and lead citrate and observed on a Philips CM12 transmission electron microscope. Images were captured with a Gatan Retractable MultiScan Camera using Digital Micrograph software.

Measurement of cartilage matrix synthesis

Assays of cartilage matrix synthesis using patellae were performed essentially as previously described (25, 26). Briefly, patellae of mice were dissected away from surrounding soft tissue and incubated overnight in media [serum-free low glucose 50:50 DMEM:F12 media with 0.1% BSA, 100 U/ml penicillin/streptomycin (Life Technologies, Inc., Gaithersburg, MD), 2 mM L-glutamine, 1× GHT, 0.1 mM MEM sodium pyruvate (Life Technologies, Inc.), 20 μg/ml gentamicin (Life Technologies, Inc.), 1.25 mg/liter Amphotericin B, 10 μg/ml] with ^{35}S-sulfur (30 μCi/ml). Patellae were then washed three times with PBS and fixed overnight in 10% formalin, followed by decalcification of the underlying bone with 5% formic acid. Cartilage, dissected away from the underlying bone, was dried, weighed, placed in 500 μl of a tissue solubilizer (Solvable, Packard Instrument Co., Meriden, CT), and incubated at 60 C for 1.5 h. Scintillation fluid designed for concentrated alkaline and salt solutions (HIONIC-fluor, Packard Instrument Co.) was added (10 ml) to each tube and mixed thoroughly and [^{35}S] quantified using a scintillation counter. Levels of proteoglycan synthesis are reported as average cpm of patellae from at least five mice per group.

Microcomputed tomography (μCT)

To quantify trabecular and cortical structure, isolated femurs (n = 5/genotype) were imaged by a SCANCO Medical (Basserdorf, Switzerland) μCT40 microimaging system at 50 kV and 80 μAmp. Contiguous axial image slices were obtained with an isotropic voxel size of 16 μm. System calibration was performed with a hydroxyapatite phantom of known density (2.91 g/cm^3). The femoral cortical bone volume, total volume, and density were determined by application of an automated

image segmentation algorithm constructed from the Analyze (AnalyzeDirect Inc., Lenexa, KS) image analysis software libraries. The algorithm employs histogram and morphological filtering (27) to extract the cortex volume. A cortical bone threshold (0.77 g hydroxyapatite/cm^3), determined by histogram analysis of the data, was applied to extract potential cortex voxels found within the image data. A series of morphological operations (erode, open, conditional dilate, and close) contained within Analyze, were then applied to link cortex voxels and remove voxels of similar density found within regions of trabecular bone. The performance of the algorithm was evaluated by comparison of automated and manual segmentation results for an independent group of mouse bone samples. The automated estimates of cortex volume ($r^2 = 0.94$), total volume ($r^2 = 0.99$), and density ($r^2 = 0.96$) were highly correlated ($P < 0.01$) with manual estimates (data not shown). Morphometric analysis of the trabecular bone within the femur was performed with the SCANCO Medical (Basserdorf, Switzerland) μCT40 evaluation software. A volume of interest (VOI) was manually defined, dorsal to the patellar grove, that contained only secondary trabecular bone and marrow space. Cortical bone was excluded by placement of the VOI boundaries within the inner boundary of the cortical bone. Before image segmentation, a constrained three-dimensional (3D) Gaussian low-pass filter was applied to image data for noise suppression. A global threshold (0.49 g hydroxyapatite/cm^3) was applied to extract a binarized trabecular structure from the VOI. The trabecular segmentation threshold was chosen by visual inspection of segmentation results from a representative subset of samples. Trabecular structural characteristics were quantified by direct 3D morphometric analysis (28). Morphometric analysis by μCT has been shown to correlate with those from histomorphometry (29). Degree of anisotropy (DA) was quantitated to indicate trabecular organization. DA of trabecular bone is defined as the ratio of the maximum mean intercept length (MIL) to the minimum MIL, where MIL is the mean length between marrow/trabecular bone interfaces along a given direction (30).

Muscle function analyses

A flow-dependent analysis of muscle function was performed using a modification of our previously developed method (31). Briefly, animals were anesthetized using 2.5% isoflurane in oxygen, and maintained at 37 C using a heat lamp. The right leg was immobilized and the third right metatarsal was sutured to a Biopac TSD 105A force transducer using 4-0 silk. Two Grass E2 platinum subdermal needle electrodes were placed through the gastrocnemius at a distance of 5 mm. A 5-V DC square wave with duration of 5 msec and an interval of 500 msec was passed through the electrodes using a Biopac STIM100C stimulator and STIMISOC isolation unit. The muscle was stimulated for 10 min, rested for 10 min, and stimulated for 10 min. Tension from the force transducer was collected and plotted using a Biopac UM100A (Biopac Systems, Inc., Santa Barbara, CA) with Acknowledge software.

Platelet-endothelial cell adhesion molecule (PECAM) measurements after femoral artery ligation

The femoral artery was isolated at the level of the inguinal ligament and ligated with 7-0 silk suture (Ethicon, Somerville, NJ). The wound was closed with 4-0 silk suture (Look) and a single 7.5-mm Michel wound clip (California Surgical, Hayward, CA). Animals recovered on a warm water heating pad until ambulatory. Anti-PECAM-1, *i.e.* rat antimouse PECAM-1, IgG_{2a} (PharMingen Inc., San Diego, CA) clone MEC13.3, labeled with ^{125}I (DuPont NEN Life Science Products, Boston, MA; NEZ-033A), specifically binds a membrane-bound molecule on endothelial cells of mouse vessels (32). A nonspecific isotype control antibody (rat antimouse CD35, IgG_{2a} (PharMingen Inc.), clone 8C12) labeled with ^{131}I (DuPont NEN Life Science Products, NEZ-035A) was used to correct for any nonspecific antibody binding, vascular leakage or any blood left in the tissue. All antibodies were iodinated using the iodogen method in a ratio of 1 μg of antibody to 1 μCi of either ^{125}I or ^{131}I (33).

To measure PECAM-1 binding, a mixture of ^{125}I PECAM-1 monoclonal antibody (mAb) (10 μg) and ^{131}I nonbinding mAb (equivalent to 500,000 cpm) was diluted with PBS to a volume of 200 μl. Starting radioactivity was counted in a 2-μl sample using a Wallac Wizard γ-counter (model 1480, Perkin-Elmer, Gaithersburg, MD). Thirty micrograms of cold PECAM-1 mAb were added to the solution. The mixture was injected through the jugular vein catheter and allowed to circulate for 5 min (34) after which a blood sample was obtained from the carotid catheter to measure circulating radiolabeled antibody levels. The animal was then exsanguinated by perfusion with bicarbonate-buffered saline through the jugular catheter (6 ml) with simultaneous blood withdrawal from the carotid catheter. Bicarbonate-buffered saline was then perfused through the carotid catheter (15 ml) after severing the inferior vena cava at the thoracic level. Entire organs and muscles were collected, weighed, and the radioactivity was counted on the γ-counter.

Results

Creation of STC transgenic mice

Two (of six) male founders with highest expression of hSTC-1 in muscle but not other tissues (data not shown) were used to establish independent lines. Because STC-1 is a secreted protein, systemic levels of STC-1 were determined in STC-1 transgenic mice (n = 30) and age-matched, wild-type (WT) mice (n = 33) using an RIA (35). Serum levels of STC-1 in trangenic mice ranged from 15–35 ng/ml with a mean value of 23.48 ± 0.78 ng/ml. In contrast, STC-1 levels in WT mice were just within assay detection limits (0.2 ng/ml), ranging from 0.3–0.8 ng/ml, with a mean value of 0.47 ± 0.02 ng/ml. No significant differences in STC-1 mRNA in muscle or serum STC-1 levels between male and female mice were detected. Thus, overexpression of STC-1 in muscle was associated with elevated systemic levels of STC-1 protein.

Growth retardation in STC transgenic mice

All six STC-1 founder mice were approximately 25% smaller than WT littermates (Fig. 1). Because growth retardation was identified in multiple founders (n = 6), the phenotype of the mice was not likely due to integration of the transgene at a locus required for normal growth. Comprehensive growth curves indicated that mice hemizygous for STC-1 were significantly smaller than WT littermates by 5 d of age for the males (Fig. 1A) and 14 d of age for the females (data not shown). STC-1 transgenic mice were viable and fertile. Blood pressure, as determined by carotid cannulation, was within normal limits.

Hormone and metabolic changes in STC-1 transgenic mice

Because STC is believed to play a role in calcium and phosphate homeostasis (6, 7), we measured levels of these minerals. Ionized calcium was significantly elevated in STC-1 transgenic female founders and offspring relative to nontransgenic littermates (11.07 mg/dl STC-1 mice *vs.* 9.95 mg/dl WT mice, $P = 0.016$). Although previous studies showed that STC can stimulate phosphate reabsorption by fish and rat renal proximal tubules (7, 36), serum phosphate levels were normal in STC-1 transgenic mice (8.93 mg/dl STC-1 mice *vs.* 8.89 mg/dl WT mice, $P = 0.951$). Renal function parameters including blood urea nitrogen and creatinine were also normal. Other serologic parameters including liver enzymes—alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and γ-glutamyltransferase—as well as amylase, a pancreatic enzyme, and creatine kinase, a cytosolic enzyme with highest activity in skeletal muscle, were within normal range (data not shown). Serum hormones—calcitonin, IGF binding protein-1, T_4, and GH—

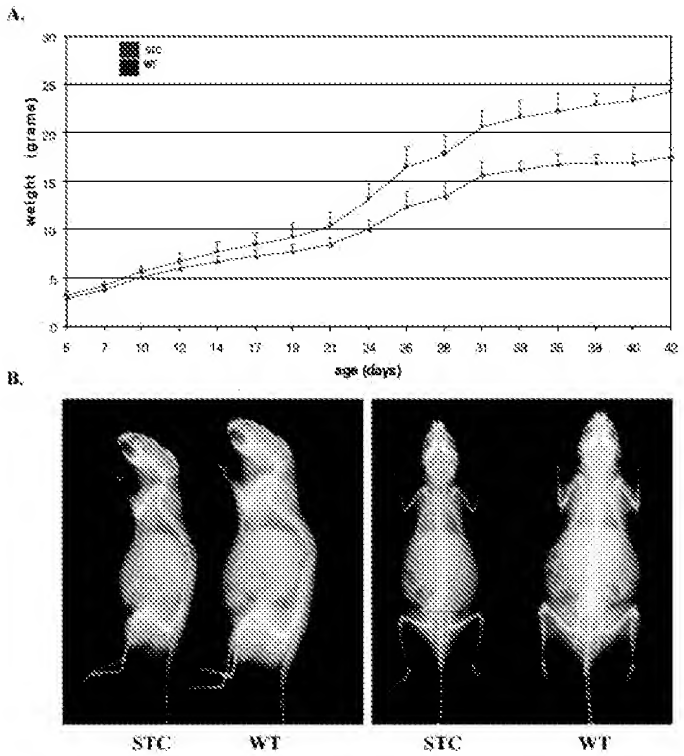


FIG. 1. Size of STC transgenic (STC) mice relative to WT littermates. A, Growth curves of male mice (n = 10/group) from 5–42 d of age. B, X-ray analysis of the skeleton of representative male transgenic (STC) or WT littermate mice from a dorsolateral (*left*) or ventrodorsal (*right*) view.

were not significantly altered in transgenic mice relative to WT mice (data not shown).

To further investigate metabolism in STC-1 mice, food intake and response to a glucose tolerance test were evaluated. At 10 wk of age, males ate 32.2% more (0.1800 ± 0.097 g/d·g body weight STC-1 *vs.* 0.1361 ± 0.0043 g/d·g body weight WT, $P = 0.015$), weighed 36% less (18.97 ± 1.10 g STC-1 *vs.* 29.67 ± 0.73 g WT $P = 0.001$), and had a 13.8% increase in oxygen consumption (8.925 ± 0.2546 ml/h·$g^{0.75}$ STC-1 *vs.* 7.8406 ± 0.2546 ml/h·$g^{0.75}$ WT $P = 0.039$). A glucose tolerance test further demonstrated metabolic differences in STC-1 transgenic mice relative to WT littermates. STC-1 transgenic mice had faster glucose clearance at 30, 45, 60, 90, and 120 min after glucose loading (data not shown). Serum-free fatty acids were normal (data not shown). Fat pad weights were decreased in female STC-1 transgenic mice relative to WT littermates even when calculated as percentage of total body weight (0.19 ± 0.09% STC-1 *vs.* 0.62 ± 0.18% WT, $P = 0.03$). Accordingly, STC-1 transgenic mice appeared leaner than their WT littermates upon dissection.

Skeletal changes STC-1 transgenic mice

As determined radiographically, the axial and appendicular skeleton was shorter in transgenics compared with WT mice (Fig. 1B). Transgenic mice had normal numbers of vertebrae (C7T13L6), but the average length of the vertebral column from the base of the atlas to the last sacral vertebra was shorter in STC-1 compared with WT mice (5.4 cm *vs.* 6.1 cm). Because longitudinal growth of bones is controlled by growth plate cartilage, histological examination of long bones was performed. In 1-d-old STC-1 transgenic mice, the structure of the femoral growth plate was histologically normal (data not shown). By 35 d of age, the growth plate of STC-1 mice was narrowed relative to WT littermates but retained normal organization and pattern of chondrocyte maturation relative to WT mice (Fig. 2A). Alcian blue staining of the growth plate showed slightly more cartilage matrix in STC-1 mice relative to WT mice.

Because STC-1 transgenic mice were substantially smaller than age-matched littermates (Fig. 1), we tested the hypothesis that STC-1 mice were merely developmentally delayed by including weight-matched (younger) mice (Fig. 2B). Long bones of STC-1 transgenic mice were 74% (femur) and 94% (tibia) the length of those of age-matched control mice, and 78% (femur) and 97% (tibia) the length of those from weight-matched control mice (data not shown). Consistent with the smaller skeleton in STC-1 mice, patellar cartilage weight was 68% that of age-matched control mice, and 90% that of weight-matched control mice (Fig. 2B, *left panel*). When corrected for this decrease in patellar cartilage weight, cartilage matrix synthesis was increased by 45% in STC-1 mice relative to age-matched controls but was 51% less than that of younger, weight-matched controls (Fig. 2B, *right panel*). As indicated by both qualitative histochemistry and quantitative assays, cartilage matrix synthesis in STC-1 mice was enhanced relative to that of age-matched controls. However, STC-1 mice did not phenocopy younger, weight-matched control mice (Fig. 2B).

Radiographs of skull bones showed altered suture morphology and decreased cellular extravasation through the center of parietal bones suggesting decreased osteoclast activity in STC-1 mice relative to WT littermates (Fig. 3A). To determine whether such changes were due to alterations in

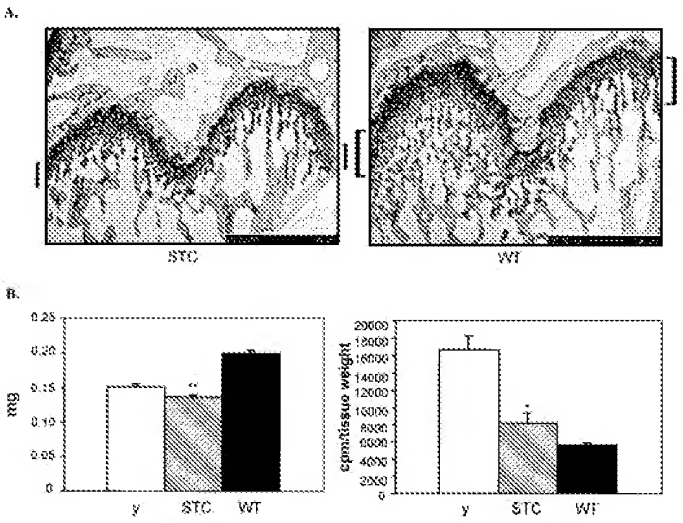


FIG. 2. Cartilage phenotype. A, Alcian *blue* staining of the growth plate (*brackets*) of 35-d-old transgenic (STC) or WT mice. B, Patellar cartilage weights (*left*) and cartilage matrix synthesis corrected for patellar cartilage weight (*right*) in transgenic mice (STC) (n = 4), age-matched controls (WT) (n = 5) and weight-matched younger mice (y) (n = 5). *, $P < 0.05$; and **, $P < 0.005$ compared with age-matched control (WT) mice using a Student's *t* test.

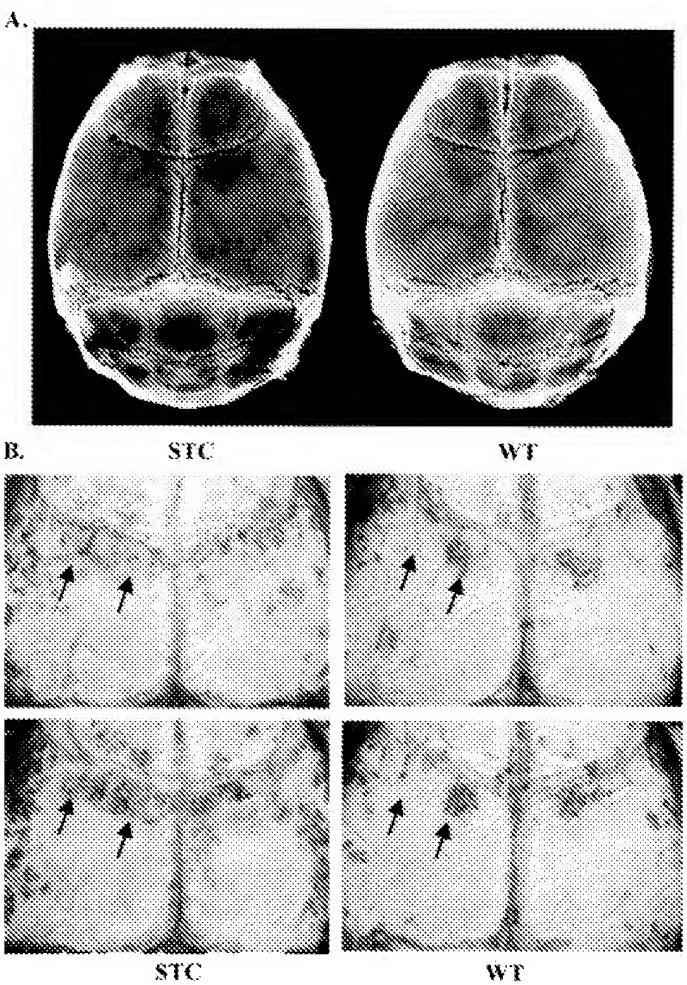


FIG. 3. The skull phenotype of STC-1 mice. A, Representative skull of an STC-1 transgenic or WT mouse. B, TRAP staining (*arrows*) for osteoclasts in parietal bones of two STC-1 transgenic mice (STC) and two WT mice.

bone-resorbing osteoclasts, we stained skulls for TRAP, an osteoclast marker. Skulls from STC-1 mice had a broader distribution of osteoclasts than did those from their WT littermates (Fig. 3B). These results are consistent with previous findings that STC affects osteoclast activity (37–39) and indicate that both intramembranous ossification (skulls) and endochondral ossification (long bones) were affected by STC-1 expression in muscle.

To determine the quality of bone formed, we examined histological sections and three-dimensional structural organization by μCT of bones. Plastic embedded sections von Kossa stained for mineral did not demonstrate striking changes in mineralization of trabecular and cortical bone in STC-1 transgenic mice (data not shown). Quantitative μCT indicated that cortical bone volume and total volume (bone and bone marrow) were reduced in STC-1 mice relative to age-matched control mice (Fig. 4A) consistent with the smaller size of STC-1 mice. However, cortical bone volume per total volume (BV/TV) was increased in STC-1 transgenic mice relative to age-matched WT mice ($P = 0.04$) (Fig. 4B), suggesting increased relative cortical bone thickness in STC-1 mice.

Despite the increase in serum calcium levels in STC-1 transgenic mice, bone mineral density of both cortical bone (Fig. 4C, *left panel*) and trabecular bone (data not shown) was not altered in STC-1 mice. However, the degree of anisotropy (DA) of trabecular bone ($P = 0.014$) was increased relative to age-matched control mice (Fig. 4, C, *right panel*, and D). This increase in DA indicated a higher directional dependence of trabecular organization corresponding to a more uniform alignment of trabeculae along the principal axis. Similarly, trabecular thickness was decreased ($P = 0.04$) in STC-1 transgenic mice (0.0516 ± 0.001) relative to WT littermates (0.0625 ± 0.004). Furthermore, although the absolute number of trabeculae was not altered (data not shown), STC-1 transgenic mice had significantly ($P = 9.4 \times 10^{-5}$) higher trabecular density (trabecular number/total volume) than their WT littermates (5.99 ± 0.28 STC-1 *vs.* 3.75 ± 0.14 WT mice). Interestingly, younger mice had increased trabecular number, thickness (data not shown) and trabecular bone volume fraction (BV/TV) (Fig. 4B) and decreased cortical bone volume fraction (BV/TV) (Fig. 4B) and decreased cortical bone mineral density (Fig. 4C, *left panel*). Thus, as was found in our cartilage assays, the skeleton of STC-1 mice did not phenocopy that of weight-matched, younger mice (Fig. 4, A–D), suggesting that the skeletal changes in STC-1 are not due merely to a developmental delay.

Given the changes in bone size and structure, we tested whether the bone-forming activity of osteoblasts was altered. No significant changes in expression of several genes—vitamin D receptor, osteopontin, osteocalcin, or bone morphogenetic protein-4—were found by quantitative real-time RT-PCR (Taqman) analysis of mRNA from bones of STC-1 and WT mice (data not shown). However, histomorphometric analysis revealed that the mineral apposition rate, a measure of osteoblast activity, was lower in both trabecular and cortical bone of STC-1 transgenic mice relative to WT littermates (1.04 μm/d STC-1 *vs.* 2.47 μm/d WT, $P = 1.9 \times 10^{-13}$) (Fig. 4E). While the rate of matrix deposition was decreased, bone mineralization in STC-1 mice was normal (Fig. 4C, *left panel*). Taken together, our data suggest that both osteoblast and osteoclast activity were decreased in STC-1 transgenic mice.

Changes in muscle function and histology

Just as endogenous STC-1 protein is present in muscle tissue (18), expression of the STC-1 transgene was directed to muscle tissue by virtue of the myosin light chain promoter (21). All muscles of STC-1 transgenic mice weighed significantly less than those of age-matched control mice and approximated the weight of muscles from weight-matched, younger mice (Fig. 5A). Thus, unlike organs such as the kidney, which weighed more in STC-1 mice than in age-matched control mice when analyzed as percentage of body weight (Fig. 5A), muscles were disproportionately smaller in STC-1 transgenic mice relative to WT littermates. To further examine skeletal muscles, electron microscopy was performed on skeletal muscle from STC-1 and WT mice. Enlarged myocyte mitochondria, with normal structure and organized cristae, were found in STC-1 transgenic mice relative to age-matched WT mice (Fig. 5B).

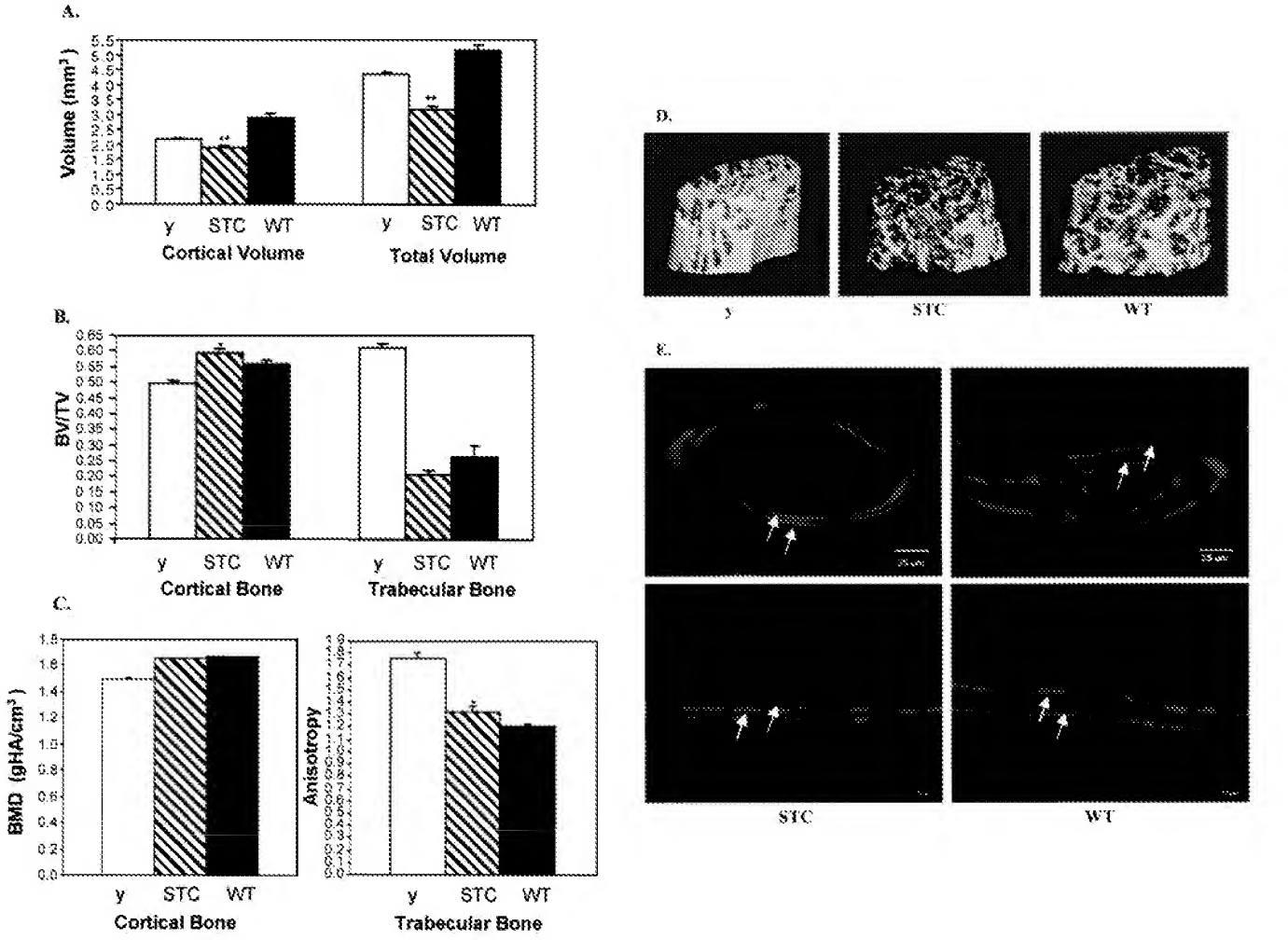


FIG. 4. Analysis of bone mineralization and structure in transgenic mice. A, Volume of cortical bone (cortical volume) and of both bone and bone marrow (total volume). B, BV/TV for cortical bone and trabecular bone. C, Bone mineral density (BMD) of cortical bone and anisotropy of trabecular bone for STC-1 transgenic mice (STC) (n = 5), age-matched (WT) (n = 5) or younger, weight-matched control mice (y) (n = 4). *, $P < 0.05$; and **, $P < 0.005$ *vs.* age-matched (WT) control mice using a Student's *t* test. D, μCT images of mouse femurs. Three-dimensional representations of trabecular bone from WT and STC-1 transgenic mice (STC) and younger (y) weight-matched control mice illustrating changes in trabecular anisotropy. E, Fluorescent micrographs of two representative sections of (*top*) trabecular and (*bottom*) cortical bone of femurs of 6-wk-old STC-1 (STC) and WT mice. Note the decrease in distance between the two labeled fronts of mineralization (*arrows*) in STC-1 mice (STC) relative to WT mice.

To quantitate muscle function, the contractile profile of muscles in response to a repetitive pulsatile electrical stimulation was determined. In this system, the early peak in response corresponds to the maximal twitch, which then slowly decreases to a plateau called the stable twitch (Ross J., L. Powell-Braxton, and S. Bunting, unpublished observation). At baseline (d 0), the slight decrease in maximal twitch response in STC-1 mice relative to WT mice was not significant (Fig. 5C, *left*, d 0). However, the stable twitch response of STC-1 transgenic mice at d 0 was significantly ($P = 0.039$) less than that of younger, approximately weight-matched WT mice (Fig. 5C, *right*, d 0).

In addition to determining baseline muscle function, we also measured the ability of muscles to respond after femoral artery ligation. In WT mice (age- and weight-matched control mice), the maximal twitch response essentially returned to baseline (97–99%) at 7 d after femoral artery ligation (Fig. 5C, *left*, d 7, *inset*). In contrast, STC-1 mice had only an approximately 80% recovery in maximal twitch response at 7 d post ligation (Fig. 5C, *left*, d 7, *inset*). In terms of the stable twitch response, WT mice showed 82–85% recovery, whereas that of STC-1 mice was only 71% at 7 d post ligation (Fig. 5C, *right*, d 7, *inset*). Thus, as indicated by both the maximal and stable twitch responses, muscle from STC-1 did not recover appropriately at 7 d after femoral artery ligation.

Changes in vascularity in STC transgenic mice

The runted phenotype of STC-1 transgenic mice could in theory be due to a vascular defect, since changes in STC-1 expression have been associated with endothelial cell differentiation (16). To test this hypothesis, we measured baseline vascular density and the angiogenic response following femoral artery ligation (32–34). STC-1 mice had a significantly

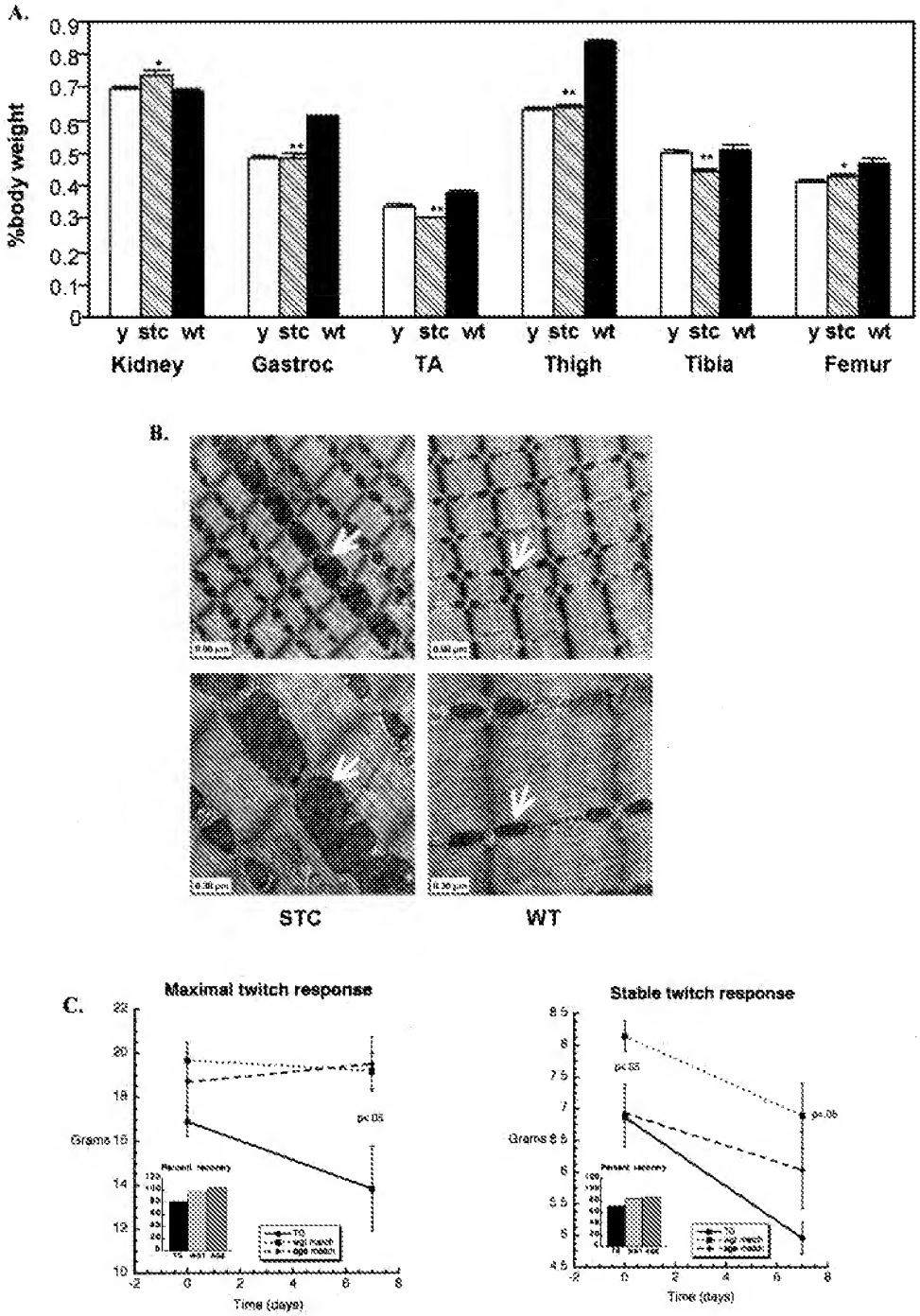


FIG. 5. Muscle mass and function in transgenic mice. A, Weight (as percent body weight) of kidney, gastrocnemius muscle (Gastroc), tibialis anterialis (TA) muscle, quadriceps femoris (Thigh), Tibia, and Femur of STC-1 (stc), age-matched control (wt) and approximately weight-matched, younger (y) mice. *, $P < 0.05$; and **, $P < 0.005$ compared with age-matched control (wt) mice using a Student's *t* test. B, EM photomicrographs of muscles from transgenic (STC) or WT mice showing enlarged mitochondria in transgenic mice. C, Maximal twitch response (*left*) and Stable twitch response (*right*) at d 0 and 7 d post ligation for STC-1 (TG) (*circle, solid line*), approximately weight-matched (wgt-match) (*square, spotted line*), and age-matched (age match) controls (*diamond, dashed line*).

higher baseline vascular density in all tissues relative to that of age-matched control mice (Fig. 6A). Compared with younger, approximately weight-matched control mice, STC-1 mice exhibited a lower vascular density in all tissues examined (Fig. 6A). Thus, as with other parameters, STC-1 transgenic mice were not identical to younger, weight-matched control mice.

Following femoral ligation, a pronounced ischemia in the lower limb of the ligated leg occurs, which is followed by a spontaneous angiogenic response. The proliferation of small blood vessels results in an increase in endothelial cell surface area, which occurs over the next 3–10 d with maximal increases in vascular density at 7 d (40). We therefore used this femoral artery ligation model to quantitate the angiogenic response to injury in STC-1 transgenic mice. Compared with age-matched WT control mice, induction of vascularity in the lower part of the left, ligated leg relative to the right (untreated) leg was significantly greater in STC-1 transgenic mice ($P < 0.05$) (Fig. 6B). Similarly, increases in vascularity in the upper leg (hamstring and femur) in response to ligation were more pronounced in STC-1 transgenic mice than in age-matched control mice. Interestingly, vascular density in-

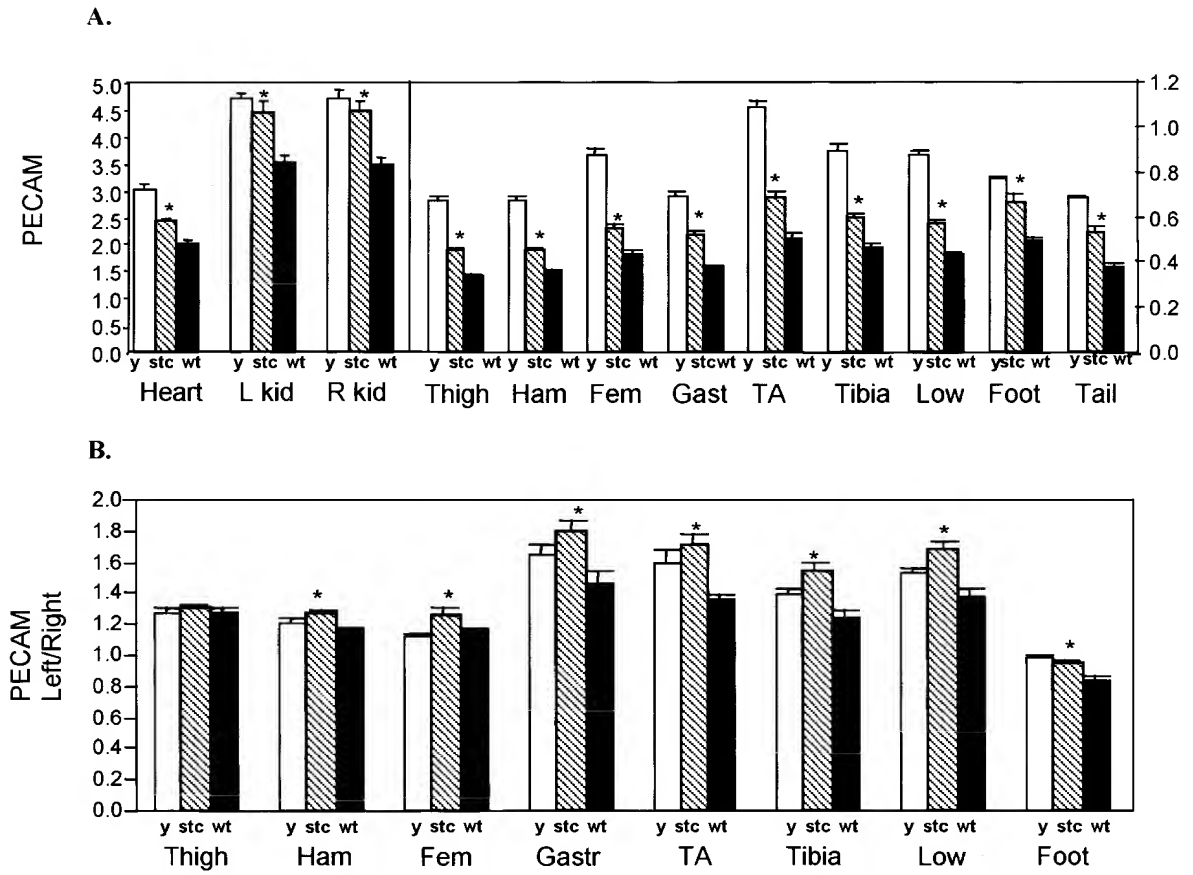


FIG. 6. Changes in vascularity in transgenic mice. Shown are measurements of PECAM, an indicator of new blood vessels in (A) Heart, kidney (L kid, R kid), Quadriceps femoris (Thigh), hamstring (Ham) muscle, femur (Fem), gastocnemius (Gast) muscle, tibialis anterialis (TA) muscle, Tibia, lower leg (Low), Foot, and Tail in STC-1 (stc), age-matched control (wt) and approximately weight-matched younger (y) mice (n = 6 for each set of mice). B, Ratios of PECAM measurements in the ligated (*Left*) *vs.* contralateral, unligated leg (*Right*) in STC-1 (stc), age-matched control (wt) and approximately weight-matched younger (y) mice (n = 8 mice/group). *, $P < 0.05$ for STC-1 compared with age-matched control (wt) mice using a Student's *t* test.

creases in STC-1 transgenic mice in response to femoral artery ligation were also higher than those of younger, approximately weight-matched control mice, even though baseline vascularity was higher in these younger mice (Fig. 6).

Discussion

Overexpression of STC-1 in transgenic mice resulted in smaller mice with distinct changes in the musculoskeletal system. The skeleton of STC-1 mice was not identical to that of younger, weight-matched controls suggesting that the phenotype of STC-1 mice was not due to a developmental delay.

Expression studies have indicated that STC-1 may play a role in bone development and patterning (18–20). Our data indicate that overexpression of STC-1 results in increased cartilage matrix synthesis and decreased bone length. Given that endogenous STC-1 is expressed at the ends of bone primordia and growing limbs (20), it is tempting to speculate that endogenous STC-1 affects the length of the cartilage anlagen, which serves as a template for subsequent bone formation. STC-1 could also act later in development as a regulator of chondrocyte maturation, as high levels of STC-1 protein are found in chondrocytes moving from the proliferative to hypertrophic zone (18). Our finding of increased cartilage matrix synthesis in STC-1 transgenic mice suggests that STC-1 stimulates chondrocytes, consistent with hypotheses that chondrocytes are targets of endogenous STC-1 (18–20).

STC-1 protein is also found in fetal trabecular osteoblasts (20) and adult mouse osteoblasts (19), and STC can regulate activity of osteoblasts (39, 41) and osteoclasts (37–39). Kinetic analyses indicated that the rate of mineral deposition by osteoblasts, but not the degree of bone mineralization, was decreased in STC-1 transgenic mice. The increases in cortical bone thickness, trabecular density, and anisotropy in the face of decreased matrix deposition by osteoblasts suggests concomitant changes in osteoclastic resorption in STC-1 mice. This hypothesis was further supported by our findings of decreased extravasation and altered osteoclast distribution in parietal bones in the skull. Thus, the changes in cortical and trabecular bone remodeling are likely due to suppression of both osteoblast and osteoclast activity by STC-1. The presence of alterations in skulls and long bones of STC-1 mice implicates STC-1 in both intramembranous and endochondral bone formation, consistent with the distribution of endogenous STC-1 protein during growth and development (18–20).

STC-1 has recently been implicated in skeletal muscle development (18). Our findings indicate that STC-1 can regulate muscle mass and function. Muscles of STC-1 mice were smaller than age-matched control mice, and muscle mitochondria were dramatically enlarged. Our assay of muscle function measuring the contractile profile of the gastrocnemius muscle indicated that STC-1 muscle had impaired ability to recover following femoral artery ligation. Our finding that STC-1 transgenic mice had a robust angiogenic response following femoral artery ligation suggest that the deficit in muscle function observed after ligation was not due to a failure to stimulate new vessel growth.

Taken together, our results indicate that STC-1 can affect muscle mass and function, perhaps at least in part by regulating muscle metabolism through alterations in mitochondria. Recently, STC protein has been found to be localized to the outer mitochondrial membranes of putative nephron target cells in fish, suggesting that STC could have direct effects on mitochondrial function (42). The enlargement of mitochondrial might be related to the protective effects that mammalian STC has on post-ischemic neuronal cells, perhaps in the transport of calcium and/or phosphate (10). It is tempting to speculate that the alterations in body composition and mitochondria are key contributors to the increased food and oxygen consumption, and faster glucose clearance in STC-1 transgenic mice.

STC-1 may also regulate endothelial cell activity. STC-1 has been implicated in endothelial cell differentiation into tube-like structures in an *in vitro* model of angiogenesis (16). STC-1 mRNA is expressed at sites of pathological angiogenesis, *i.e.* in tumors (16, 17). To evaluate a potential role for STC-1 in angiogenesis, we employed the femoral artery ligation model which provides an *in vivo* model of spontaneous angiogenesis following lower limb ischemia. In our angiogenesis experiments, we normalized values to the weight of the tissue to adjust for differences in size. Measurements of baseline vascular density strongly suggested that STC-1 mice had significantly higher capillary density in organs (heart and kidney) and tissues (muscle and bone) compared with age-matched littermates. The ability of STC-1 mice to mount a spontaneous angiogenic response following an ischemic insult was significantly increased relative to age-matched WT mice. These changes could be due to direct effects of STC-1 on endothelial cells and their environment, consistent with previous studies showing up-regulation of STC-1 upon endothelial cell differentiation (16). The finding that STC-1 mice showed a larger increase in vascularity after femoral ligation than did younger, approximately weight-matched controls supports the conclusion that the phenotype of STC-1 mice is not due merely to a developmental delay.

Angiogenesis is also a critical component of organ and tissue growth. STC-1 mice exhibited a vascular density greater than age-matched control mice, suggesting that a perfusion deficit is unlikely to explain their runted phenotype. The lack of obvious changes in wound healing following the femoral ligation surgery supports our data indicating robust vascular responses in STC-1 transgenic mice. The dramatic decrease in body weight and size in STC-1 transgenic mice is likely due, at least in part, to effects of STC-1 on the musculoskeletal system.

While this manuscript was in preparation, analysis of transgenic mice overexpressing STC-1 by virtue of the metallothionein I minimal promoter indicated a role for STC-1 in growth and reproduction (43). Consistent with their data, and despite our use of a different promoter, our transgenic mice (which had 5–100 times lower serum levels of STC-1) were smaller and had higher serum calcium levels than WT mice. No changes in reproduction were observed with our mouse lines when we mated male transgenic mice with female WT mice, in accordance with their findings that reproductive function of male mice was not affected (43). The phenotype of our STC-1 transgenic mice similarly indicate a role for STC-1 in growth and calcium homeostasis and further identify and characterize the musculoskeletal changes induced by STC-1 overexpression.

In summary, our results indicate how STC-1 can affect muscle mass and function and bone size and structure. Future studies will help elucidate the relationship between STC-1 and other factors regulating development of the musculoskeletal system.

Acknowledgments

The authors thank Sharon Erickson and her lab for generating and genotyping STC-1 mice, Patti Tobin and Robin E. Taylor for histology support, and Frank Peale for helpful discussions.

Received December 20, 2001. Accepted May 6, 2002.

Address all correspondence and requests for reprints to: Dr. Ellen Filvaroff, Department of Molecular Oncology, Genentech, Inc., MS 72B, 1 DNA Way, South San Francisco, California 94080. E-mail: filvarof@gene.com.

Support from the Kidney Foundation of Canada and The Canadian Institutes of Health Research (to G.F.W.) is acknowledged.

References

1. **Wagner GF, Hampong M, Park CM, Copp DH** 1986 Purification, characterization, and bioassay of teleocalcin, a glycoprotein from salmon corpuscles of Stannius. Gen Comp Endocrinol 63:481–491
2. **Lafeber FP, Flik G, Wendelaar Bonga SE, Perry SF** 1988 Hypocalcin from Stannius corpuscles inhibits gill calcium uptake in trout. Am J Physiol 254: R891–R896
3. **Wagner GF, Dimattia GE, Davie JR, Copp DH, Friesen HG** 1992 Molecular cloning and cDNA sequence analysis of coho salmon stanniocalcin. Mol Cell Endocrinol 90:7–15
4. **Chang AC, Janosi J, Hulsbeek M, de Jong D, Jeffrey KJ, Noble JR, Reddel RR** 1995 A novel human cDNA highly homologous to the fish hormone stanniocalcin. Mol Cell Endocrinol 112:241–247
5. **Varghese R, Wong CK, Deol H, Wagner GF, DiMattia GE** 1998 Comparative analysis of mammalian stanniocalcin genes. Endocrinology 139:4714–4725
6. **Madsen KL, Tavernini MM, Yachimec C, Mendrick DL, Alfonso PJ, Buergin M, Olsen HS, Antonaccio MJ, Thomson AB, Fedorak RN** 1998 Stanniocalcin: a novel protein regulating calcium and phosphate transport across mammalian intestine. Am J Physiol 274:G96–G102
7. **Wagner GF, Vozzolo BL, Jaworski E, Haddad M, Kline RL, Olsen HS, Rosen CA, Davidson MB, Renfro JL** 1997 Human stanniocalcin inhibits renal phosphate excretion in the rat. J Bone Miner Res 12:165–171
8. **Worthington RA, Brown L, Jellinek D, Chang AC, Reddel RR, Hambly BD, Barden JA** 1999 Expression and localisation of stanniocalcin 1 in rat bladder, kidney and ovary. Electrophoresis 20:2071–2076
9. **Zhang KZ, Westberg JA, Paetau A, von Boguslawsky K, Lindsberg P, Erlander M, Guo H, Su J, Olsen HS, Andersson LC** 1998 High expression of stanniocalcin in differentiated brain neurons. Am J Pathol 153:439–445
10. **Zhang K, Lindsberg PJ, Tatlisumak T, Kaste M, Olsen HS, Andersson LC** 2000 Stanniocalcin: a molecular guard of neurons during cerebral ischemia. Proc Natl Acad Sci USA 97:3637–3642
11. **Lal A, Peters H, St. Croix B, Haroon ZA, Dewhirst MW, Strausberg RL, Kaanders JH, van der Kogel AJ, Riggins GJ** 2001 Transcriptional response to hypoxia in human tumors. J Natl Cancer Inst 93:1337–1343
12. **Franzen AM, Zhang KZ, Westberg JA, Zhang WM, Arola J, Olsen HS,**

Andersson LC 2000 Expression of stanniocalcin in the epithelium of human choroid plexus. Brain Res 887:440–443
13. **Deol HK, Varghese R, Wagner GF, Dimattia GE** 2000 Dynamic regulation of mouse ovarian stanniocalcin expression during gestation and lactation. Endocrinology 141:3412–3421
14. **Stasko SE, DiMattia GE, Wagner GF** 2001 Dynamic changes in stanniocalcin gene expression in the mouse uterus during early implantation. Mol Cell Endocrinol 174:145–149
15. **Stasko SE, Wagner GF** 2001 Stanniocalcin gene expression during mouse urogenital development: a possible role in mesenchymal-epithelial signalling. Dev Dyn 220:49–59
16. **Kahn J, Mehraban F, Ingle G, Xin X, Bryant JE, Vehar G, Schoenfeld J, Grimaldi CJ, Peale F, Draksharapu A, Lewin DA, Gerritsen ME** 2000 Gene expression profiling in an in vitro model of angiogenesis. Am J Pathol 156: 1887–1900
17. **Fujiwara Y, Sugita Y, Nakamori S, Miyamoto A, Shiozaki K, Nagano H, Sakon M, Monden M** 2000 Assessment of stanniocalcin-1 mRNA as a molecular marker for micrometastases of various human cancers. Int J Oncol 16:799–804
18. **Jiang WQ, Chang AC, Satoh M, Furuichi Y, Tam PP, Reddel RR** 2000 The distribution of stanniocalcin 1 protein in fetal mouse tissues suggests a role in bone and muscle development. J Endocrinol 165:457–466
19. **Yoshiko Y, Son A, Maeda S, Igarashi A, Takano S, Hu J, Maeda N** 1999 Evidence for stanniocalcin gene expression in mammalian bone. Endocrinology 140:1869–1874
20. **Stasko SE, Wagner GF** 2001 Possible roles for stanniocalcin during early skeletal patterning and joint formation in the mouse. J Endocrinol 171:237–248
21. **Shani M** 1985 Tissue-specific expression of rat myosin light-chain 2 gene in transgenic mice. Nature 314:283–286
22. **Stewart TA, Clift S, Pitts-Meek S, Martin L, Terrell TG, Liggitt D, Oakley H** 1992 An evaluation of the functions of the 22-kilodalton (kDa), the 20-kDa, and the N-terminal polypeptide forms of human growth hormone using transgenic mice. Endocrinology 130:405–414
23. **Hogan B, Beddington R, Constantini F, Lacy E** 1994 Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Press
24. **Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR** 1987 Bone histomorphometry: standardization of nomenclature, symbols, and units. J Bone Miner Res 2:595–610
25. **Verschure PJ, Joosten LA, van der Kraan PM, Van den Berg WB** 1994 Responsiveness of articular cartilage from normal and inflamed mouse knee joints to various growth factors. Ann Rheum Dis 53:455–460
26. **Cai L, Yin JP, Starovasnik MA, Hogue D, Hillan KJ, Mort JS, Filvaroff EH** 2001 Pathways by which interleukin 17 induces articular cartilage breakdown *in vitro* and *in vivo*. Cytokine 16:10–21
27. **Jain AK** 1989 Fundamentals of digital image processing. Englewood Cliffs, NJ: Prentice Hall
28. **Hildebrand T, Laib A, Muller R, Dequeker J, Ruegsegger P** 1999 Direct Three-dimensional morphometric analysis of human cancellous bone: microstructual data from spine, femur, iliac crest, and calcaneus. J Bone Miner Res 14:1167–1174
29. **Muller R, Van Campenhout H, Van Damme B, Van der Perre G, Dequeker J, Hildebrand T, Ruegsegger P** 1998 Morphometric analysis of human bone biopsies: a quantitative structural comparison of histological sections and micro-computed tomography. Bone 23:59–66
30. **Harrigan TP, Mann RW** 1984 Characterization of microstructural anisotropy in orthotropic material using a second rank tensor. J Mater Sci 19:761–767
31. **Walder CE, Errett CJ, Bunting S, Lindquist P, Ogez JR, Heinsohn HG, Ferrara N, Thomas GR** 1996 Vascular endothelial growth factor augments muscle blood flow and function in a rabbit model of chronic hindlimb ischemia. J Cardiovasc Pharmacol 27:91–98
32. **Vecchi A, Garlanda C, Lampugnani MG, Resnati M, Matteucci C, Stoppacciaro A, Schnurch H, Risau W, Ruco L, Mantovani A, Dejana E** 1994 Monoclonal antibodies specific for endothelial cells of mouse blood vessels, their application in the identification of adult and embryonic endothelium. Eur J Cell Biol 63:247–254
33. **Eppihimer MJ, Russell J, Langley R, Vallien G, Anderson DC, Granger DN** 1998 Differential expression of platelet-endothelial cell adhesion molecule-1 (PECAM-1) in murine tissues. Microcirculation 5:179–188
34. **Panes J, Perry MA, Anderson DC, Manning A, Leone B, Cepinskas G, Rosenbloom CL, Miyasaka M, Kvietys PR, Granger DN** 1995 Regional differences in constitutive and induced ICAM-1 expression *in vivo*. Am J Physiol 269:H1955–H1964
35. **De Niu P, Radman DP, Jaworski EM, Deol H, Gentz R, Su J, Olsen HS, Wagner GF** 2000 Development of a human stanniocalcin radioimmunoassay: serum and tissue hormone levels and pharmacokinetics in the rat. Mol Cell Endocrinol 162:131–144
36. **Lu M, Wagner GF, Renfro JL** 1994 Stanniocalcin stimulates phosphate reabsorption by flounder renal proximal tubule in primary culture. Am J Physiol 267:R1356–R1362
37. **Lafeber FP, Schaefer HI, Herrmann-Erlee MP, Wendelaar Bonga SE** 1986 Parathyroid hormone-like effects of rainbow trout Stannius products on bone resorption of embryonic mouse calvaria *in vitro*. Endocrinology 119:2249–2255
38. **Lafeber FP, Herrmann-Erlee MP, Flik G, Wendelaar Bonga SE** 1989 Rainbow trout hypocalcin stimulates bone resorption in embryonic mouse calvaria in vitro in a PTH-like fashion. J Exp Biol 143:165–175
39. **Yoshiko Y, Kosugi T, Koide Y** 1996 Effects of a synthetic N-terminal fragment of stanniocalcin on the metabolism of mammalian bone *in vitro*. Biochim Biophys Acta 1311:143–149
40. **Isner JM** 2000 Tissue responses to ischemia: local and remote responses for preserving perfusion of ischemic muscle. J Clin Invest 106:615–619
41. **Yoshiko Y, Maeda S, Aubin JE** 2000 Stanniocalcin is a novel autocrine/paracrine factor necessary for bone formation. J Bone Miner Res 15:S503
42. **Amemiya Y, Marra LE, Reyhani N, Youson JH** 2002 Stanniocalcin from an ancient teleost: a monomeric form of the hormone and a possible extracorpuscular distribution. Mol Cell Endocrinol 188:141–150
43. **Varghese R, Gagliardi AD, Bialek PE, Yee SP, Wagner GF, DiMattia GE** 2002 Over-expression of human stanniocalcin affects growth and reproduction in transgenic mice. Endocrinology 143:868–876

ELSEVIER

Molecular and Cellular Endocrinology 213 (2004) 167–172

MCE
Molecular and Cellular Endocrinology

www.elsevier.com/locate/mce

Co-localization of stanniocalcin-1 ligand and receptor in human breast carcinomas

Christopher R. McCudden [a], Agata Majewski [a], Subrata Chakrabarti [b], Graham F. Wagner [a,*]

[a] *Department of Physiology and Pharmacology, Faculty of Medicine and Dentistry, The University of Western Ontario, London, Ont., Canada N6A 5C1*

[b] *Department of Pathology, The University of Western Ontario, London, Ont., Canada N6A 5C1*

Received 17 September 2003; accepted 3 October 2003

Abstract

Stanniocalcin-1 (STC1) is a new polypeptide hormone that has metabolic effects on target cell mitochondria. Recent studies have shown that the STC1 gene is upregulated in primary breast tumors and co-expressed with the estrogen receptor. In this report we have demonstrated the histological co-localization of STC1 and its receptor in invasive and non-invasive human mammary gland ductal carcinomas. Analysis of 58 malignant breast biopsies revealed that STC1 and its receptor co-localized to cancer cells in 91% of cases. The study therefore reveals that in breast carcinomas STC1 signals in an autocrine feedback loop and opens up the possibility that it may be sequestered by neoplastic cells in much the same manner as it is by non-malignant cells. The data further supports the notion that STC1 plays a role in breast cancer and that it may prove to be a novel diagnostic and prognostic marker, and potential therapeutic target.

Keywords: Breast cancer; Stanniocalcin-1; Ligand; Receptor

1. Introduction

The stanniocalcin (STC) family of polypeptide hormones, comprising STC1 and STC2, are widely expressed in mammalian tissues. Whereas the functions of STC2 are less certain, STC1 has recently been shown to have stimulatory effects on target cell mitochondrial electron transport (McCudden et al., 2002). Equally intriguing are recent data derived from transgenic mice where STC1 over-expression results in increased oxygen consumption and metabolic wasting, in combination with hyperphagia and a dwarf phenotype (Filvaroff et al., 2002; Varghese et al., 2002). A number of recent reports have now implicated STC1 and STC2 in the carcinogenic process (Bouras et al., 2002; Charpentier et al., 2000; Fujiwara et al., 2000; Garber et al., 2001; Gruvberger et al., 2001; Ismail et al., 2000; Lal et al., 2001; Liang et al., 1992; Watanabe et al., 2002; Welcsh et al., 2002). The majority of these studies have used differential display or DNA microarray analysis and focussed on the identification of genes with altered patterns of expression. While the steady-state levels of STC1 mRNA are increased in variety of tumor types (Liang et al., 1992; Fujiwara et al., 2000; Garber et al., 2001; Lal et al., 2001), STC2 has been primarily implicated in breast cancer as an estrogen responsive gene (Charpentier et al., 2000; Gruvberger et al., 2001; Bouras et al., 2002; Wilson et al., 2002). Thus it appears that STC1 is involved not only in cell metabolism, but also in the carcinogenic process and/or subsequent tumor growth.

Most recently it has been demonstrated that STC1 and STC2 mRNAs and their proteins are both induced in human breast cancer cells (Bouras et al., 2002; Wascher et al., 2003). The induction of STC genes in breast cancer and the fact that they encode secreted proteins, raises the intriguing question as to whether or not these hormones are destined to act back on breast cancer cells in an autocrine feedback loop, or are, in fact, targeted to surrounding adipose and stromal tissue. We have recently developed the means for identifying STC1 receptors at the histological level using a fusion protein of STC1 and placental alkaline phosphatase

* Corresponding author. Tel.: +1-519-661-3966; fax: +1-519-661-3827.
E-mail address: graham.wagner@fmd.uwo.ca (G.F. Wagner).

0303-7207/$ – see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2003.10.042

(McCudden et al., 2002). Therefore, in this report we have used this methodology to localize the STC1 ligand and its receptor at the cellular level in a wide range of human breast cancer biopsies.

2. Materials and methods

Sections of fixed and paraffin embedded human breast cancer biopsies from 58 affected individuals were obtained from The London Health Sciences Center, Department of Pathology, London, Ontario. Only tissues in which informed patient consent had been provided beforehand in the form of a signed statement were used in the study. Tissues were accompanied by a detailed surgical pathology report, including a gross description of the tissue, microscopic description of tumor type and histological grade of the cancer. Patients ranged in age from 36 to 84 years, with an average of 60.7 ± 13 years (S.D.). Tumor size averaged $13.7 \pm 2.9\,cm^3$ (S.E.M.) and tumor SBR grade averaged 2.2 ± 0.09 (S.E.M.). Only 20% of the patients (11/58) showed evidence of blood or lymphatic invasion as indicated by lymph node involvement, whereas ductal carcinoma in situ (DCIS) was identified in 50% of the cases examined (29/58). Of the 58 specimens, 42 had also been screened for estrogen receptors, thereby allowing a comparison with the expression of STC ligand and receptor (Table 1).

Immunocytochemistry and in situ ligand binding were performed on fixed tissue sections to localize STC immunoreactive protein and STC binding sites/receptors at the cellular level. Northern blot analysis was also carried out on normal and malignant mammary tissue to determine if the STC gene was more preferentially expressed in breast cancer.

Table 1
Comparison of ISLB and ICC stain grading of human breast cancer biopsies

Stain grade[a]	STC receptors	STC protein
+++	8/58	10/58
++	26/58	18/58
+	19/58	25/58
−	5/58	5/58
Total (positive)	53/58	53/58

[a] Stain grade was qualitatively assessed by the relative intensity of the stain.

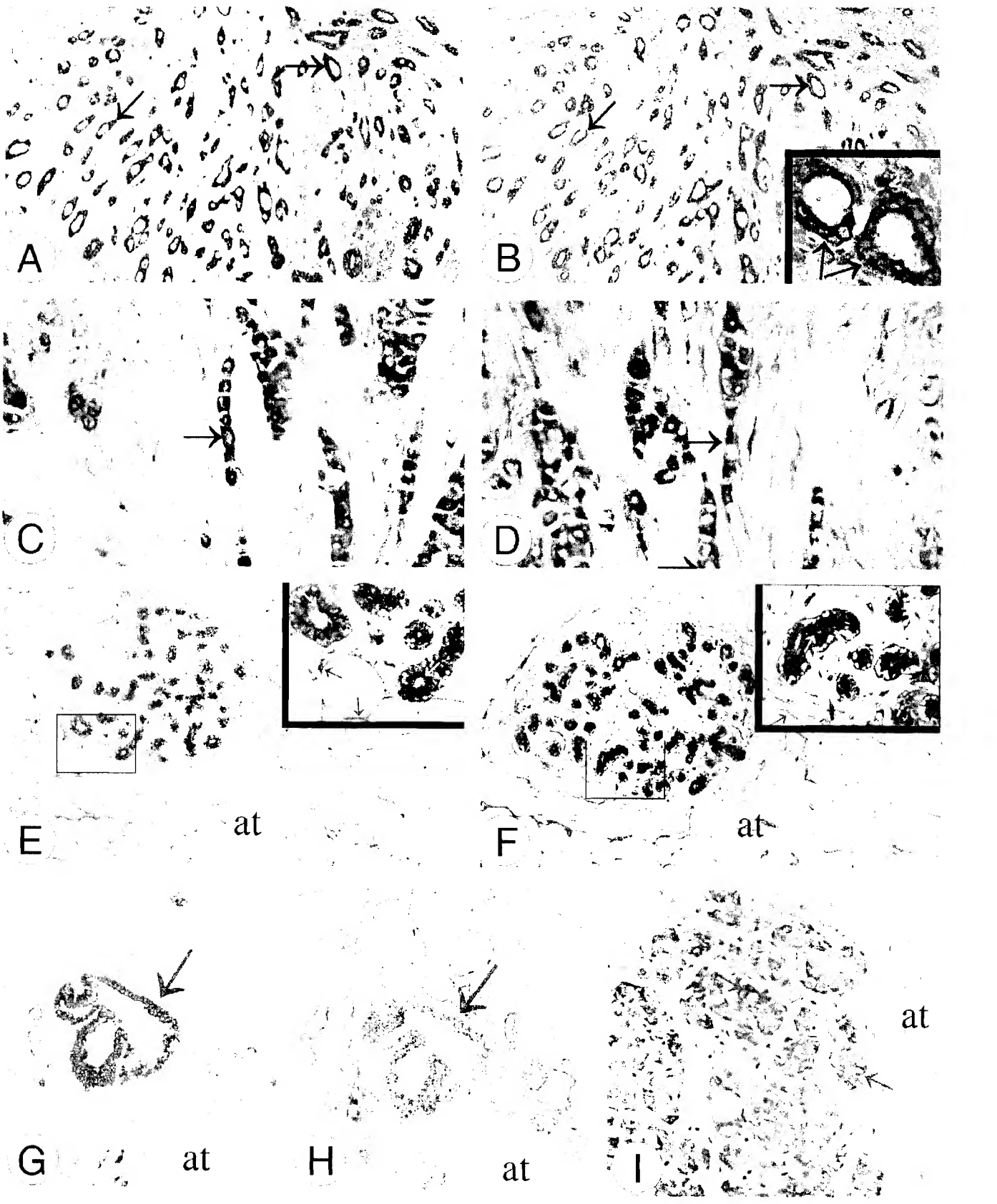
2.1. In situ ligand binding (ISLB)

A STC-alkaline phosphatase (STC-AP) fusion protein was generated and used for in situ ligand binding (ISLB) as previously described (McCudden et al., 2002). The specificity of the procedure for the STC1 receptor has previously been established by showing that ligand binding is not displaceable by mammalian hormones such as GH, PRL, FSH, LH and hCG, or STC2, but readily displaceable with recombinant STC1 (McCudden et al., 2002). Tissue sections were first equilibrated in HBHA buffer (Hanks solution with 1% BSA and 20 mM HEPES) and then incubated in 0.5 nM STC-AP dissolved in HBHA for 90 min at room temperature (controls received STC-AP plus 1 μM hSTC). Sections were then washed in HBHA and fixed for 1 min in 60% acetone, 3% formaldehyde, 20 mM HEPES, pH 7.5. Endogenous AP activity was destroyed by heating the tissues at 65 °C for 60 min. Color development of ligand-associated AP activity was then initiated by the addition of AP detection buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.33 mg/ml NBT and 0.17 mg/ml BCIP). Slides were washed in distilled water to stop the reaction, dehydrated and mounted.

2.2. Immunocytochemistry (ICC)

Immunocytochemistry was carried out as previously described using a polyclonal antiserum generated against recombinant human STC1. This antiserum has been well characterized in terms of its specificity for STC1 (Deol et al., 2001). Tissue sections were pretreated with 10% normal goat serum in diluent buffer (10 mM Tris, pH 7.5, containing 150 mM NaCl) to prevent non-specific staining, followed by an overnight incubation at 4 °C with hSTC antiserum (1:250 dilution). The sites of antigen–antibody binding were visualized using the Avidin–Biotin peroxidase method (Vectastain, Vector Laboratories Inc., Burlingame, CA) and the chromagen 3,3′-diaminobenzidine tetrachloride (Sigma, St. Louis, MO). Primary antiserum containing 1 μM hSTC was used as a control method for non-specific binding. Slides were washed in distilled water to stop the staining reaction, dehydrated and mounted. Slides were photographed with a SPOT2 digital camera. Grading of staining intensity following ICC and ISLB were performed essentially as described for the HER-2 oncogene (Birner et al., 2001).

Fig. 1. Co-localization of STC1 and its receptor in invasive and ductal carcinoma in situ, and normal human mammary gland. Panels A–D are invasive carcinomas; panels E–F are of ductal carcinoma in situ; panels G–I are of normal mammary gland. Panels A/C/E: immunocytochemical (ICC) localization of STC1 ligand, as indicated by the dark brown staining (arrows). Staining is exclusively over the cytoplasm of cancer cells. Some staining of adipocytes in surrounding adipose tissue (at) is also evident in panel E (red arrows). Panels B/D/F: in situ ligand binding (ISLB) localization of STC1 binding sites/receptors. Specific binding, as indicated by the purple staining (arrows), was exclusively over the cytoplasm of cancer cells (white and yellow arrows) and adipocytes (red arrows) of surrounding adipose tissue (at). In the case of ductal carcinoma in situ, both ligand (panel E, white arrows) and receptor (panel F, yellow arrows) exhibited a tendency to be concentrated apically, adjacent to the ductal lumen. Panels G/H: ductal epithelium in non-malignant mammary gland following ICC (G) and ISLB (H). Note that there is good co-localization of ligand and receptor in the epithelial cells (arrows). Panel I: ICC localization of STC ligand in non-malignant mammary gland alveolar cells (arrows). The ICC and ISLB control slides revealed no tissue staining and are not shown (online version of Fig. 1 is in colour).


A
B
C
D
E
at
F
at
G
at
H
at
I
at

2.3. Northern blotting

Total RNA was extracted from frozen biopsies of 10 normal and 20 malignant mammary glands using standard methods and resolved on 1% agarose denaturing gels (50 μg total RNA/lane). The RNA was then transferred to nitrocellulose membrane and sequentially probed with human STC and 18S ribosomal RNA cDNA probes as previously described (Deol et al., 2001). The blots were exposed to X-ray film and the resulting bands quantified by scanning densitometry.

3. Results

The results of the immunocytochemical and in situ ligand binding staining are shown in Fig. 1 (panels A–F). Both STC1 and its receptor co-localized in 91% of the biopsies examined (53/58), and virtually all cancerous cells were positively stained for both ligand and receptor. This was true in the case of invasive carcinomas (Fig. 1; panels A–D), as well as in the case of ductal carcinoma in situ (Fig. 1; panels E–F). In all instances, the cell cytoplasm was homogeneously stained for both ligand and receptor. There was no evidence of nuclear staining. In the case of ductal carcinoma in situ, however, there was a tendency for ligand and receptor to be preferentially concentrated on the apical margins of the cells, adjacent to the ductal lumen (Fig. 1; inset of panels E–F). Weak staining of the surrounding adipocytes was also evident.

In a limited number of cases we examined the patterns of ligand and receptor localization in normal mammary gland. Here we observed intense co-localization of ligand and receptor in ductal epithelial cells (Fig. 1; panels G–H) and to a lesser extent in alveolar tissue (Fig. 1; panel I). As in the case of the cancer cells, the staining in normal mammary gland was purely cytoplasmic in nature. The high incidence of positive staining for STC ligand and receptor (91%) compared favorably with the proportion that were positively stained for estrogen and progesterone receptors (71%), but less favorably so with indices of vascular invasion (20%) and DCIS status (50%).

The results of the Northern blot analysis are shown in Fig. 2. Our findings here revealed that of the 20 tumor specimens analyzed, the majority (90%) contained detectable STC transcript, although signal intensity did vary widely in some cases. In contrast, STC transcript was undetectable in the majority of normal mammary gland specimens we examined. However, an important factor that was difficult to control for in this case was the physical nature of the specimens. Whereas the majority of tumors consisted of cancerous epithelial cells embedded within a stromal matrix, the biopsies of normal mammary gland usually contained only small amounts of ductal epithelia and a much higher proportion of adipose tissue. That being said, this does not detract from the central observation that both invasive and non-invasive carcinoma cells were highly positive for both STC and its receptor.

4. Discussion

Using a recently developed in situ ligand binding assay for localizing STC1 binding sites and standard immunocytochemistry for ligand localization, we have mapped the distribution of STC and its binding sites/receptors in 58 individual breast carcinomas. Our data showed that cancer cells were the only cells possessing both STC and STC receptors (except for surrounding adipocytes), suggesting mechanistically, that in the malignant mammary gland STC is signaling in an autocrine feedback loop. In tissues such as the ovary, STC is known to operate via both paracrine and endocrine pathways. Within the kidney, however, an autocrine signaling pathway has been identified in the collecting ducts, whose cells make STC and also possess high affinity STC receptors (Deol et al., 2001; McCudden et al., 2002). Hence, this is the first instance that we are aware

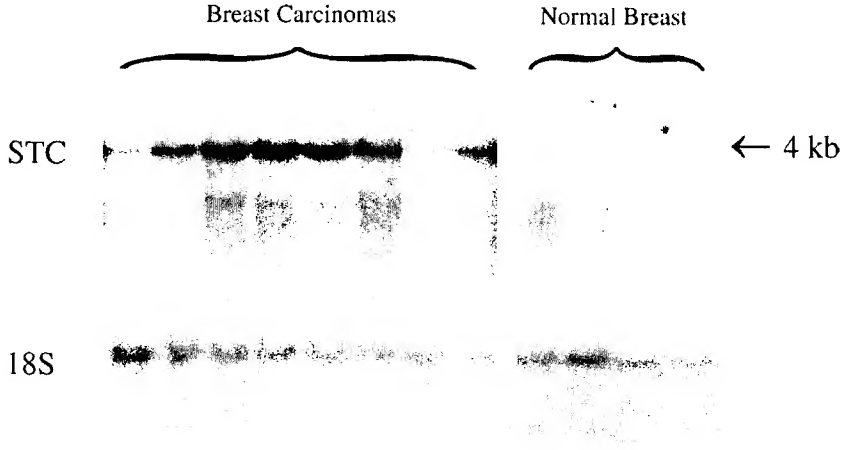


Fig. 2. Northern blot analysis of normal breast and tumor biopsies. Total RNA (50 μg) from individual tumors and normal mammary tissue was probed with a [^{32}P]-labeled human STC1 cDNA. This revealed the presence of a 4 kilobase (kb) STC1 transcript that was most prevalent in tumor tissue.

of whereby STC appears to be operating via an autocrine loop in a malignant setting. Our results also lend further support to the recent reports by others on the presence of STC1 mRNA and immunoreactive protein in breast tumors (Bouras et al., 2002; Wascher et al., 2003).

While the role of STC1 in breast cancer cells remains to be determined, there is evidence for its involvement in a variety of phenomena related to tumor formation and growth. For example, in non-cancerous cells STC1 gene expression is increased in response to hypoxia, angiogenesis, and cellular growth and differentiation (Gerritsen et al., 2002; Lal et al., 2001; Stasko and Wagner, 2001). Furthermore, STC1 over-expression has pronounced inhibitory effects on somatic growth in mice (Filvaroff et al., 2002; Varghese et al., 2002). In the case of somatic growth, part of the response may be due to STC having an effect upon cell metabolism. Recent studies have shown that in tissues such as liver and kidney, STC1 is heavily sequestered by target cell mitochondria where it increases electron transport. Furthermore, the majority of cells targeted in this manner are known to be highly metabolically active (Stasko and Wagner, 2001; McCudden et al., 2002). Similarly, frank mitochondrial hypertrophy is evident in the muscle cells of STC1 transgenic mice, presumably as a result of STC hyper-signaling (Filvaroff et al., 2002). Hence, given the tendency for STC1 to be targeted to highly active cells and the high metabolic demand of proliferating cancer cells, it is possible that STC1 is similarly involved in regulating the metabolic activity of cancer cells. Although most of the increased metabolism in cancer cells is attributable to changes in glycolysis rather than aerobic respiration (the Warburg effect) (Dang and Semenza, 1999), STC1 stimulation of mitochondrial respiration could serve as an added energy source in tumor cells. Alternatively, it is possible that STC1 is involved in other mitochondrial processes related to tumorigenesis. For example, a role in preventing cellular apoptosis should not be ruled out, especially in view of the fact that STC1 is cytoprotective under conditions of hyperosmotic and hypoxic stress (Sheikh-Hamad et al., 2000; Zhang et al., 2000).

It is noteworthy that two additional reports on STC1 in breast cancer appear to contradict those of the present study. Both of these found that STC1 was undetectable in primary tumors, but readily detected in normal ductal epithelium (Liang et al., 1992; Welcsh et al., 2002). In this regard, tumor source and/or variability are the likely causes of this discrepancy. While our Northern blotting revealed that the levels of STC1 gene expression were reasonably high in most tumors as compared to normal human breast tissue, a small number of the tumors we examined (5–10%) had low or undetectable levels of expression. Thus STC1 may be absent in a subset of breast tumors.

STC1 and its receptor were also found to be present in all of the samples that were estrogen receptor-positive (30/30). This is in complete agreement with previous findings, where STC1 and STC2 protein and mRNA were highly correlated with ER levels (Bouras et al., 2002). Although there is as yet no evidence demonstrating estrogen regulation of the STC receptor, it is now evident that in breast tumors STC1 receptors and estrogen receptors are typically co-expressed. On the other hand, the high incidence of positive staining for STC and its receptor contrasted with the much lower incidence of lymph node involvement (20%) and DCIS status (50%).

The abundance of STC1 receptor, mRNA and protein in tumors could potentially make it a valuable diagnostic and prognostic marker of breast cancer. Currently there are several markers used for the diagnosis, prognosis or staging of metastases, such as carcinoembryonic antigen, estrogen and progesterone receptors, and epidermal growth factor (Osborne et al., 1980; Osborne, 1998). However, none of these markers can be used alone for all three purposes on account of their lack of specificity or sensitivity to breast cancer alone. A recent report shows that STC1 mRNA in bone marrow and blood of breast cancer patients is significantly correlated with tumor size and several indices of metastasis (Wascher et al., 2003). Our findings also indicate that STC and its receptor are so highly prevalent in breast cancer cells (91% of biopsies) that both could prove to be excellent alternative markers of the disease. Future research should now be focused on defining the role of STC in normal and malignant breast tissue.

Acknowledgements

We gratefully acknowledge Janette Botz and the London Health Sciences Centre Tumour Bank for providing the tissues used in this study. This work was supported by funds from The Canadian Institutes of Health Research and The Kidney Foundation of Canada awarded to G.F.W. and a Natural Sciences and Engineering Council of Canada Postgraduate Scholarship awarded to C.R.M.

References

Birner, P., Oberhuber, G., Stani, J., Reithofer, C., Samonigg, H., Hausmaninger, H., Kubista, E., Kwasny, W., Kandioler-Eckersberger, D., Gnant, M., Jakesz, R., 2001. Evaluation of the United States Food and Drug Administration-approved scoring and test system of HER-2 protein expression in breast cancer. Clin. Cancer Res. 7, 1669–1675.

Bouras, T., Southey, M.C., Chang, A.C., Reddel, R.R., Willhite, D., Glynne, R., Henderson, M.A., Armes, J.E., Venter, D.J., 2002. Stanniocalcin 2 is an estrogen-responsive gene coexpressed with the estrogen receptor in human breast cancer. Cancer Res. 62, 1289–1295.

Charpentier, A.H., Bednarek, A.K., Daniel, R.L., Hawkins, K.A., Laflin, K.J., Gaddis, S., MacLeod, M.C., Aldaz, C.M., 2000. Effects of estrogen on global gene expression: identification of novel targets of estrogen action. Cancer Res. 60, 5977–5983.

Dang, C.V., Semenza, G.L., 1999. Oncogenic alterations of metabolism. Trends Biochem. Sci. 24, 68–72.

Deol, H., Stasko, S.E., De Niu, P., James, K.A., Wagner, G.F., 2001. Post-natal ontogeny of stanniocalcin gene expression in rodent kidney and regulation by dietary calcium and phosphate. Kidney Int. 60, 2142–2152.

Filvaroff, E.H., Guillet, S., Zlot, C., Bao, M., Ingle, G., Steinmetz, H., Hoeffel, J., Bunting, S., Ross, J., Carano, R.A., Powell-Braxton, L., Wagner, G.F., Eckert, R., Gerritsen, M.E., French, D.M., 2002. Stanniocalcin 1 alters muscle and bone structure and function in transgenic mice. Endocrinology 143, 3681–3690.

Fujiwara, Y., Sugita, Y., Nakamori, S., Miyamoto, A., Shiozaki, K., Nagano, H., Sakon, M., Monden, M., 2000. Assessment of Stanniocalcin-1 mRNA as a molecular marker for micrometastases of various human cancers. Int. J. Oncol. 16, 799–804.

Garber, M.E., Troyanskaya, O.G., Schluens, K., Petersen, S., Thaesler, Z., Pacyna-Gengelbach, M., van de Rijn, M., Rosen, G.D., Perou, C.M., Whyte, R.I., Altman, R.B., Brown, P.O., Botstein, D., Petersen, I., 2001. Diversity of gene expression in adenocarcinoma of the lung. Proc. Natl. Acad. Sci. U.S.A. 98, 13784–13789.

Gerritsen, M.E., Soriano, R., Yang, S., Ingle, G., Zlot, C., Toy, K., Winer, J., Draksharapu, A., Peale, F., Wu, T.D., Williams, P.M., 2002. In silico data filtering to identify new angiogenesis targets from a large in vitro gene profiling data set. Physiol. Genomics 12, 13–20.

Gruvberger, S., Ringner, M., Chen, Y., Panavally, S., Saal, L.H., Borg, A., Ferno, M., Peterson, C., Meltzer, P.S., 2001. Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. Cancer Res. 61, 5979–5984.

Ismail, R.S., Baldwin, R.L., Fang, J., Browning, D., Karlan, B.Y., Gasson, J.C., Chang, D.D., 2000. Differential gene expression between normal and tumor-derived ovarian epithelial cells. Cancer Res. 60, 6744–6749.

Lal, A., Peters, H., St Croix, B., Haroon, Z.A., Dewhirst, M.W., Strausberg, R.L., Kaanders, J.H., van der Kogel, A.J., Riggins, G.J., 2001. Transcriptional response to hypoxia in human tumors. J. Natl. Cancer Inst. 93, 1337–1343.

Liang, P., Averboukh, L., Keyomarsi, K., Sager, R., Pardee, A.B., 1992. Differential display and cloning of messenger RNAs from human breast cancer versus mammary epithelial cells. Cancer Res. 52, 6966–6968.

McCudden, C.R., James, K.A., Hasilo, C., Wagner, G.F., 2002. Characterization of mammalian stanniocalcin receptors: mitochondrial targeting of ligand and receptor for regulation of cellular metabolism. J. Biol. Chem. 277, 45249–45258.

Osborne, C.K., 1998. Steroid hormone receptors in breast cancer management. Breast Cancer Res. Treat. 51, 227–238.

Osborne, C.K., Yochmowitz, M.G., Knight III, W.A., McGuire, W.L., 1980. The value of estrogen and progesterone receptors in the treatment of breast cancer. Cancer 46, 2884–2888.

Sheikh-Hamad, D., Rouse, D., Yang, Y., 2000. Regulation of stanniocalcin in MDCK cells by hypertonicity and extracellular calcium. Am. J. Physiol. 278, F417–F424.

Stasko, S.E., Wagner, G.F., 2001. Possible roles for stanniocalcin during early skeletal patterning and joint formation in the mouse. J. Endocrinol. 171, 237–248.

Varghese, R., Gagliardi, A.D., Bialek, P.E., Yee, S.P., Wagner, G.F., Dimattia, G.E., 2002. Overexpression of human stanniocalcin affects growth and reproduction in transgenic mice. Endocrinology 143, 868–876.

Wascher, R.A., Huynh, K.T., Giuliano, A.E., Hansen, N.M., Singer, F.R., Elashoff, D., Hoon, D.S.B., 2003. Stanniocalcin-1: a novel molecular blood and bone marrow marker for human breast cancer. Clin Cancer Res. 9, 1427–1435.

Watanabe, T., Ichihara, M., Hashimoto, M., Shimono, K., Shimoyama, Y., Nagasaka, T., Murakumo, Y., Murakami, H., Sugiura, H., Iwata, H., Ishiguro, N., Takahashi, M., 2002. Characterization of gene expression induced by RET with MEN2A or MEN2B mutation. Am. J. Pathol. 161, 249–256.

Welcsh, P.L., Lee, M.K., Gonzalez-Hernandez, R.M., Black, D.J., Mahadevappa, M., Swisher, E.M., Warrington, J.A., King, M.C., 2002. BRCA1 transcriptionally regulates genes involved in breast tumorigenesis. Proc. Natl. Acad. Sci. U.S.A. 99, 7560–7565.

Wilson, K.S., Roberts, H., Leek, R., Harris, A.L., Geradts, J., 2002. Differential gene expression patterns in HER2/neu-positive and -negative breast cancer cell lines and tissues. Am. J. Pathol. 161, 1171–1185.

Zhang, K.Z., Lindsberg, P.J., Tatlisumak, T., Kaste, M., Olsen, H.S., Andersson, L.C., 2000. Stanniocalcin: A molecular guard of neurons during cerebral ischemia. PNAS 97, 3637–3642.

Stanniocalcin-1: A Novel Molecular Blood and Bone Marrow Marker for Human Breast Cancer[1]

Robert A. Wascher, Kelly T. Huynh, Armando E. Giuliano, Nora M. Hansen, Frederick R. Singer, David Elashoff, and Dave S. B. Hoon[2]

Department of Molecular Oncology [R. A. W., K. T. H., D. S. B. H.] and Joyce Eisenberg-Keefer Breast Center [A. E. G., N. M. H., F. R. S.], John Wayne Cancer Institute, Santa Monica, California 90404, and Department of Biomathematics, University of California Los Angeles School of Medicine, Los Angeles, California 90024 [D. E.]

ABSTRACT

Purpose: **Very few tumor molecular markers have been identified that are highly specific for breast cancer cells when applied to blood and bone marrow (BM). Stanniocalcin (*STC*)-1 is a recently discovered human gene that has been implicated in cellular calcium homeostasis and resistance to hypoxia and is located on chromosome 8p in a region associated with amplification in breast cancer. We investigated *STC-1* mRNA as a potential molecular marker for detection of breast cancer metastasis in the blood and BM.**

Experimental Design: **Using the reverse transcriptase-PCR and electrochemiluminescence detection assay to assess for *STC-1* mRNA expression, we evaluated 7 breast cancer cell lines, 34 primary breast cancer tumors, and the BM of 71 patients and the blood of 58 patients with American Joint Committee on Cancer stage 0–IV breast cancer.**

Results: **In this cohort of primarily early-stage breast cancer patients, the detection of *STC-1* mRNA in the BM and blood significantly correlated with multiple histopathological prognostic factors, including primary tumor size, number of positive lymph nodes, T stage, M stage, N stage, and overall American Joint Committee on Cancer stage. *STC-1* mRNA was not detected in the blood or BM of volunteers without cancer. *In situ* hybridization studies with a *STC-1* antisense RNA probe also confirmed *STC-1* mRNA expression in breast cancer cell lines and primary breast tumors.**

Conclusions: ***STC-1*** **is proposed as a novel, specific, and clinically useful molecular marker for detecting occult breast cancer cells in the BM and blood.**

INTRODUCTION

AJCC[3] stage I and II breast cancer patients who have undergone curative surgery and show no evidence of LN or distant metastasis still have a long-term recurrence rate of 30–50% (1–3). The BM and cortical bone are the most common sites of breast cancer metastasis, followed by visceral and soft tissue sites (4). Breast cancer patients who ultimately develop systemic metastases after complete surgical resection may remain clinically free of disease for years before disease recurrence finally reaches the threshold of current clinical and radiographic methods of detection. The earliest possible diagnosis of breast cancer, both primary and recurrent, is of considerable clinical importance, and it can be used to make treatment decisions while tumor burden is low, and when patients are most likely to respond to adjuvant therapy. Breast cancer is known to hematogenously spread to the BM and other distant sites. However, a clinically useful molecular marker has not yet been validated for the detection of occult breast cancer metastases in the blood and BM.

A highly conserved homologue of the fish glycoprotein STC has recently been identified in humans (5, 6). In fish, the product of this gene is secreted by the organ of Stannius in response to hypercalcemia, resulting in the inhibition of calcium absorption from the gills and gut and increased resorption of phosphorus by the kidney (7). *STC-1* has been mapped to 8p11.2-p21, a region of gene amplification described in breast cancer and of putative tumor suppressor genes thought to play a role in breast cancer (8–11). For this reason, we evaluated *STC-1* as a potential molecular tumor marker for breast cancer. The human STC gene (*STC-1*) consists of 4 exons and spans 13 kb, with an open reading frame of 741 bp. The 4-kb human *STC-1* mRNA transcript codes for a 247-amino acid glycoprotein (M_r ~30,000). The *STC-1* protein shares 70–80% amino acid sequence similarity with the homologous STC proteins of several bony fish species and 98% amino acid sequence similarity to mouse STC protein (6, 12–14).

Although its physiological function in humans is unclear at this time, recombinant human *STC-1* is physiologically active in both mammals and fish, causing decreased calcium uptake and increased phosphate absorption when applied to the duodenum

Received 6/24/02; revised 12/9/02; accepted 12/10/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by United States Army Grant DAMD17-01-1-0279, the University of California 7WB-0021-University of California Breast Cancer Research Program; the Ben B. and Joyce E. Eisenberg Foundation (Los Angeles, CA), the Gonda Foundation (Los Angeles, CA), and the Fashion Footwear Association of New York (New York, NY).
[2] To whom requests for reprints should be addressed, at Department of Molecular Oncology, John Wayne Cancer Institute, 2200 Santa Monica Boulevard, Santa Monica, CA 90404-2302. E-mail: hoond@jwci.org.

[3] The abbreviations used are: AJCC, American Joint Committee on Cancer; BM, bone marrow; ECL, electrochemiluminescence; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; ISH, *in situ* hybridization; LN, lymph node; PBL, peripheral blood lymphocyte; RT-PCR, reverse transcriptase-PCR; *STC*, *stanniocalcin*; TBR, Tris(2,2′-bipyridine) ruthenium(II); ECL U, ECL unit(s); TNM, tumor-node-metastasis.

of rats and swine and decreased renal phosphate excretion after injection into goldfish and rats (13–15). Limited expression of the *STC-1* gene in humans has been identified in the renal tubules, ovary, prostate, pancreas, small intestine, and thyroid (5, 16). In contrast to its effects in fish, in which STC appears to act as an endocrine hormone, *STC-1* appears to function in an autocrine/paracrine fashion to regulate calcium and phosphate uptake and excretion in mammals (13–15). However, *STC-1* has no significant amino acid or nucleotide homology to the major mammalian calcium regulation endocrine hormones, calcitonin and parathyroid hormone (17).

The RT-PCR assay has been used to detect occult metastatic tumor cells in breast cancer patients, based on its ability to detect tumor-associated mRNA as a surrogate of tumor cells (18). At the present time, breast cancer mRNA tumor markers are relatively few in number, and many of them are also expressed by normal cells found in the blood, BM, and LNs (19–22). The detection of occult circulating tumor cells in the blood and BM of patients who are clinically free of residual breast cancer is important in identifying patients with subclinical metastatic disease. Breast cancer patients often experience a prolonged clinical disease-free interval between initial diagnosis/treatment and clinical recurrence. The ability to detect early recurrence in the blood or BM may allow for adjuvant treatment to be instituted when the patient's tumor burden is still quite minimal. Monitoring of patient response to systemic adjuvant therapy using specific tumor mRNA markers would be similarly useful, allowing for the early modification of adjuvant treatment to an alternative regimen if persistent occult tumor cells are detected in the blood or BM. Therefore, the identification of new and specific RT-PCR markers for detecting occult metastatic breast cancer cells in the blood and BM is necessary.

Based on its location in a region known to contain breast cancer-related genes and its apparent role in cellular calcium homeostasis, we investigated *STC-1* mRNA as a potential molecular marker for the detection of breast cancer cells in the blood and BM using RT-PCR and quantitative ECL analysis and real-time quantitative RT-PCR. Preliminary evaluation of *STC-1* in our laboratory revealed no expression of *STC-1* mRNA in the blood or BM of healthy volunteers without cancer.[4] Therefore, we assessed the blood and BM of patients with breast cancer for evidence of *STC-1* mRNA expression in this study and correlated *STC-1* expression with known clinicopathological prognostic factors. ISH was also used to validate *STC-1* mRNA expression in breast cancer cell lines and in paraffin-embedded breast cancer tumor sections.

MATERIALS AND METHODS

Cell Lines. The breast cancer cell lines BT-20, MCF-7, HBL-100, T-47D, MDA-MB-231, 734B, JM992Br, PM277Br, and the choriocarcinoma cell line JAR were used. The cell lines were grown in RPMI 1640 (Gemini Bioproducts, Calabasas, CA) plus 10% heat-inactivated FCS, penicillin (Life Technologies, Inc., Grand Island, NY), and streptomycin (Life Technologies, Inc.) in 75-cm^2 flasks.

[4] Unpublished data.

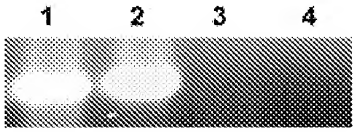


Fig. 1 *STC-1* RT-PCR product (172 bp) derived from BT-20 and JM992Br breast cancer cell lines. *Lane 1,* BT-20; *Lane 2,* JM992Br; *Lane 3,* negative control (reverse transcriptase/H_2O); *Lane 4,* negative control (PCR/H_2O).

Patient Specimens. Breast tumor surgical specimens and blood were obtained after obtaining informed patient consent, and in consultation with the surgeon and pathologist. All tissues were collected and dissected under stringent sterile conditions to prevent RNA contamination. Institutional review board approval was obtained for the use of human subject blood, BM, and tumor specimens from breast cancer patients and normal healthy donors. Blood was collected from 58 female patients with AJCC stage 0–IV breast cancer. Bilateral percutaneous needle iliac crest BM aspirations were performed on an additional 71 AJCC stage I–III breast cancer patients under general anesthesia after obtaining informed consent and just before resection of their primary breast tumors and/or axillary LNs. Blood specimens obtained from 44 healthy donors with no history of cancer were used as negative controls in the blood RT-PCR/ECL assay. Frozen mononuclear cells isolated from adult normal healthy donor BM (n = 3) were purchased from BioWhittaker, Inc. (Walkersville, MD) and used as negative controls in the BM RT-PCR/ECL assay. All patient specimens were coded before being assessed, and individuals performing RT-PCR assays were blinded regarding all patients' clinical status.

Ten ml of blood were collected in sodium citrate-containing tubes from breast cancer patients and healthy volunteer donors, as described previously (19). PBLs from whole blood and mononuclear cells from BM were isolated using Purescript RBC lysis buffer (Gentra Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. TRI Reagent (Molecular Research Center, Cincinnati, OH) was used to isolate total RNA from the cell lines, surgical tumor specimens, BM, and PBLs as described previously (19). The concentration, purity, and amount of total RNA were determined by UV spectrophotometry. Purified RNA was then treated with RQ1 RNase-free DNase (Promega, Madison, WI) to eliminate any genomic DNA contamination.

RT-PCR Primer and Probe Synthesis. Biotinylated oligonucleotide primers for PCR and a TBR chelate-labeled hybridization probe for use in an ECL PCR cDNA product detection assay were synthesized by Gemini Biotech (Alachua, FL) and Midland Certified Reagent Company (Midland, TX), respectively, as described previously (23, 24). To avoid amplification and detection of genomic DNA, primer and probe sequences were designed to span 1 intron and designed for optimal use in the PCR and ECL assay systems. *STC-1* primer sequences used were as follows: 5′ primer, 5′-TGAGGTCGTCCAGCTGCCCAATC-3′ (exon 3); and 3′ primer, 5′-GGCACATGTGTCTGTCTGCAGGATG-3′ (exon 4); with a resulting cDNA product of 172 bp (Fig. 1).

All PCR products were then detected using a quantitative ECL probe-based assay (IGEN International, Inc., Gaithersburg, MD), as described previously (24, 25). The TBR-labeled ECL probe was designed in our laboratory and synthesized by Midland Certified Reagent Company. The sequence for the *STC-1* oligonucleotide probe was 5′-TCCAGCAGGCTTCGGACAAG-3′.

PCR primers and a TaqMan fluorescent probe for a new real-time quantitative PCR assay were also designed in our laboratory and were used to evaluate *STC-1* mRNA expression levels in breast cancer cell lines. The *STC-1* real-time PCR primer sequences used were as follows: 5′ primer, 5′-CACTTCTCCAACAGATACT-3′ (exon 3); and 3′ primer, 5′-CATGTTAGGCCCAATTTTC-3′ (exon 4). The probe was conjugated with 6-carboxyfluorescein at the 5′ end and hexachloro-6-carboxyfluorescein at the 3′ end. Probe sequence was 5′-CCTGCTGGAATGTGATGAAGACAC-3′. The real-time PCR primers and TaqMan probe were synthesized by MWG-Biotech (High Point, NC) and Biosource International (Camarillo, CA), respectively. A reference standard for *STC-1* mRNA copy number quantification was developed in our laboratory. Briefly, *STC-1* PCR product was generated from breast cancer cell lines known to express *STC-1*. After confirmation of the expected PCR product size (111 bp) by agarose gel electrophoresis, the *STC-1* bands were excised from the gel and purified (Qiagen, Inc., Valencia, CA). The purified *STC-1* PCR product was ligated into a plasmid vector, which was then used to transform vector-competent *Escherichia coli* cells according to the manufacturer's instructions for chemical transformation (Topo TA Cloning kit; Invitrogen Life Technologies, Inc., Carlsbad, CA). Confirmation of successful *STC-1* PCR product ligation into the plasmid vector was obtained after restriction enzyme digestion. Serial 10-fold dilutions of the *STC-1*-ligated plasmids were then performed using molecular biology grade water and used as copy number standards during all real-time PCR reactions assays.

RT-PCR/ECL Assay. The integrity of all RNA specimens was verified by performing RT-PCR/ECL to detect the mRNA of the housekeeping gene coding for porphobilinogen deaminase. Primer and probe sequences and PCR conditions for porphobilinogen deaminase have been described previously (26). Tissue processing, RNA extraction, RT-PCR assay set-up, and post-PCR product analysis were carried out in separate designated rooms to avoid contamination. Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase according to the manufacturer's instructions (Promega). All reverse transcription reactions were carried out with oligo(dT) priming, as described previously (27). The same amount of RNA was used in all reactions for all samples in the study, including the control samples. Hybaid (Middlesex, United Kingdom) thermocyclers were used to perform all PCR reactions. The optimized PCR conditions for *STC-1* analysis consisted of 1 cycle of denaturing at 95°C for 5 min; followed by 35 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min; and a final product extension step at 72°C for 10 min.

In each experiment set-up, samples of RT-PCR reagents without mRNA, H_2O alone, and normal healthy donor PBL mRNA were used in all RT-PCR steps and in ECL analysis as negative controls. Tumor cell lines known to be RT-PCR for *STC-1* expression were used in all assays as positive controls.

ECL detection assays with the Origen Analyzer (IGEN International, Inc.) were used for detection of *STC-1* PCR cDNA products using a TBR-labeled hybridization probe, as described previously (24, 26). Negative controls used with the ECL assay included the H_2O controls for the reverse transcription and PCR reactions as well as ECL assay bead, probe, and buffer solutions without cDNA products. The *STC-1* RT-PCR assay was considered positive when the ECL assay results (in ECL U) were greater than 3 SDs above the mean ECL values of BM and blood from healthy volunteer donors. Assays were repeated at least twice to verify results.

Real-Time Quantitative PCR Assay. All RNA samples were isolated from breast cancer cell lines and quantified as described above. The synthesis of cDNA from total cellular RNA was performed as described above. The integrity of all RNA specimens was verified by performing quantitative real-time PCR to detect the mRNA of the housekeeping gene coding for glyceraldehyde-3-phosphate dehydrogenase.

A total of 1 μg of RNA was used for all samples in the study, including the positive control samples. The iCycler thermocycler (Bio-Rad, Hercules, CA) was used to perform all real-time quantitative PCRs. The optimized PCR conditions for real-time *STC-1* analysis consisted of 1 cycle of denaturing at 95°C for 10 min, followed by 40 cycles at 54°C for 1 min and 72°C for 1 min, and a final product extension step at 72°C for 10 min. Threshold cycle numbers were calculated during PCR amplification for each *STC-1* plasmid standard, and a standard curve was then created using the iCycler data analysis software. Based on this standard curve, absolute copy numbers were then calculated for all experimental PCR samples after 40 cycles of amplification.

ISH Assay. MCF-7 and BT-20 breast cancer cell lines were grown to 30–40% confluence on culture well glass slides, using the culture conditions described above, and then rapidly fixed with methanol and acetone washes. Paraffin-embedded archived primary breast cancer tissues, both IDCs and ILCs, were cut into 5-μm sections, deparaffinized, rehydrated, and prepared for ISH. RNA antisense probes against *STC-1* were constructed using the *STC-1* PCR cDNA product from a cell line known to express *STC-1* (Ambion, Inc., Austin, TX).

ISH with the *STC-1* antisense probe was then performed on both tumor cell lines and human breast tumor specimens (Ambion, Inc.). A digoxigenin-labeled β-actin antisense RNA probe (Ambion, Inc.) was used as a positive control, and hybridization buffer without RNA probe was used as a negative control. After ISH, all slides were then rinsed with Tris-buffered saline buffer [50 mM Tris-HCl (pH 7.5) and 150 mM NaCl] and developed with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt solution at −30°C (Boehringer Mannheim, Mannheim, Germany) until adequate colorization was obtained. The color reaction was then stopped with nuclease-free water, and the slides were mounted with an aqueous mounting solution. All slides were then viewed with a microscope and photographed with a digital camera.

Statistical Analysis. To investigate the association between established breast cancer clinicopathological characteristics and the presence of tumor-associated *STC-1* mRNA, χ^2 and

Table 1 *STC-1* RT-PCR results

Specimen	RT-PCR positive (%)
Breast cancer cell lines[a]	8/8 (100)
Primary breast tumors	31/34 (91)
Breast cancer patient blood	12/58 (21)
Breast cancer patient BM	18/71 (25)

[a] One cell line = choriocarcinoma.

Table 2 *STC-1* real-time RT-PCR of breast cancer cell lines

Breast cancer cell line	Threshold cycle	*STC-1* mRNA copy no. (per 250 ng of RNA)
JM992Br	28.18	934
T-47D	29.41	395
BT-20	27.99	1,070
MCF-7	23.14	31,200
HBL-100	30.26	219

Wilcoxon rank-sum tests were used. $P \leq 0.05$ was considered statistically significant.

RESULTS

RT-PCR/ECL Assay Detection Sensitivity. To assess the potential sensitivity of the *STC-1* RT-PCR/ECL assay for detection of tumor cells in blood and BM under established optimal assay conditions, an *in vitro* model was set up by serially diluting tumor cells in healthy donor PBLs. The breast cancer cell lines MCF-7 and BT-20, both known to express *STC-1* mRNA, were serially diluted in healthy donor PBLs to determine the detection sensitivity of the RT-PCR assay. Tumor cells could be detected at concentrations of 1–5 tumor cells in 10^7 PBLs with the *STC-1* RT-PCR/ECL assay.

RT-PCR Analysis of Cell Lines and Primary Breast Tumors. The expression of *STC-1* in cell lines and primary breast tumors was evaluated by RT-PCR. The cell lines MCF-7, 734B, BT-20, T-47D, MDA-MB-231, JM992Br, PM277Br, and JAR were assessed for *STC-1* mRNA marker expression by non-real-time RT-PCR/ECL analysis. All seven breast cancer cell lines and JAR expressed *STC-1* mRNA. In addition, 34 primary breast tumor specimens from patients with AJCC stage I–III breast cancer were tested for *STC-1* mRNA expression. A total of 31 of these 34 primary tumor specimens (91%) were found to be positive for the *STC-1* mRNA breast tumor marker (Table 1). The mean ECLU value for the 31 *STC-1*-positive breast tumors was 183,381, whereas the mean ECLU value for the three *STC-1*-negative tumors was 7,550.

Quantitative real-time RT-PCR was performed to determine *STC-1* mRNA copy level in the breast cancer cell lines JM992Br, T-47D, BT-20, MCF-7, and HBL-100. These cell lines were found to express variable *STC-1* mRNA copy levels, ranging from 219 to 31,200 copies per 250 ng of cellular RNA (Table 2).

***STC-1* Expression in BM.** Breast cancer cell detection in BM was evaluated using the *STC-1* RT-PCR/ECL assay because occult metastasis in the BM has been shown to be an independent and negative prognostic factor in breast cancer (28–34). BM specimens obtained from three healthy donors with no history of cancer were negative for *STC-1* expression under optimal assay conditions. Patient clinical status was classified according to AJCC stage at the time of BM collection as follows: 38 of 71 patients (54%) had AJCC stage I breast cancer; 30 of 71 patients (42%) had AJCC stage II disease, and 3 of 71 patients (4%) had AJCC stage III disease (Table 3). The histopathological status of the axillary LNs, based on H&E staining results, was also evaluated as a component of patient clinical status. A single patient with a LN that was negative by H&E stain but positive for cytokeratin-positive cells by immunohistochemistry alone was therefore not included in the LN status analysis. The mean ECL value for the three BM specimens from healthy donors was 7,330 ECL U. Overall, 18 of 71 patients (25%) had detectable *STC-1* mRNA in their BM. The mean ECL U value for the 18 *STC-1*-positive BM specimens was 19,400, whereas the mean ECL U value for the 53 *STC-1*-negative BM specimens was 7,880. Five of 38 stage I patients (13%), 11 of 30 stage II patients (37%), and 2 of 3 stage III patients (67%) had detectable *STC-1* mRNA in the BM.

Table 3 Correlation between *STC-1* RT-PCR BM marker status and patient characteristics

Clinical characteristics	*STC-1* BM marker status: Negative (n = 53)	*STC-1* BM marker status: Positive (n = 18)	P
No. of LNs			
Mean	0.55	2.61	<0.002[a]
SD	1.4	3.8	
Tumor size			
Mean	1.70	2.61	0.05[a]
SD	1.4	2.3	
T			
T_{1a}	5	0	
T_{1b}	12	0	
T_{1c}	21	8	<0.01[a]
T_2	13	6	
T_3	1	2	
N[b]			
N_0	42	7	
N_1	10	11	<0.01[c]
AJCC			
I	33	5	
II	19	11	
III	1	2	<0.01[a]

[a] Wilcoxon rank-sum test.

[b] One patient excluded due to LN micrometastasis detected by immunohistochemistry but not by H&E staining.

[c] χ^2 test.

***STC-1* Expression in BM and Clinical Correlation.** Clinicopathological data were reviewed and analyzed for the 71 breast cancer patients who underwent RT-PCR of their BM. Age, tumor grade, tumor type, Bloom-Richardson grading score, size of primary tumor, TNM stage, overall AJCC stage, number of involved axillary LNs, and estrogen/progesterone receptor status were evaluated relative to *STC-1* expression using χ^2 and Wilcoxon rank-sum tests in all 71 patients. The presence of *STC-1* mRNA in the BM significantly correlated with the number of involved LNs ($P < 0.002$, Wilcoxon rank-sum test), size of the primary tumor ($P = 0.05$, Wilcoxon rank-sum test), T stage ($P < 0.01$, Wilcoxon rank-sum test), N stage ($P < 0.01$, χ^2 test), and overall AJCC stage ($P < 0.001$,

Table 4 Correlation between *STC-1* RT-PCR blood marker status and patient characteristics

Clinical characteristics	*STC-1* blood marker status[a]		P^b
	Negative (n = 46)	Positive (n = 12)	
No. of LNs			
Mean	1.3	4.3	0.06[c]
SD	3.0	7.9	
Tumor size			
Mean	2.06	3.45	**<0.03**[c]
SD	1.4	2.2	
M stage			
M_0	43	7	**0.003**[d]
M_1	2	5	
AJCC stage			
I	21	3	**0.03**[c]
II	16	3	
III	6	2	
IV	2	4	

[a] Patient numbers do not equal 58 for all clinical characteristics due to incomplete patient data from referring institution.

[b] T stage and N stage not included (not significant). Significant values shown in bold.

[c] Wilcoxon rank-sum test.

[d] χ^2 test.

Wilcoxon rank-sum test; Table 3). The number of involved axillary LNs, TNM stage, and overall AJCC stage are all primary clinicopathological determinants of breast cancer prognosis and are surrogates of disease outcome. All other clinicopathological parameters failed to reach significance with respect to *STC-1* marker status of the BM. Due to the small number of clinical disease-related events in this group of predominantly early-stage patients, correlation of *STC-1* BM marker status with disease recurrence and death was not feasible. Long-term follow-up will be required to further define the precise prognostic significance of detection of *STC-1* mRNA in the BM.

***STC-1* Expression in Blood.** The detection of occult circulating breast tumor cells in the peripheral blood has been demonstrated previously using molecular assays (35, 36). Although the prognostic significance of this finding remains unclear, we have shown previously that the detection of breast tumor mRNA markers in the blood correlates with AJCC stage of disease (37).

Blood was obtained from 44 healthy donors with no history of cancer. *STC-1* mRNA was not detected in any of the normal donor blood specimens under the optimal assay conditions. The mean *STC-1* RT-PCR assay value for the blood of the 44 healthy donors without cancer was 5,934 ECL U.

Blood was obtained from 58 breast cancer patients. Patient clinical status was classified according to the AJCC stage at the time of blood collection as follows: 1 of 58 patients had ductal carcinoma *in situ* (AJCC stage 0); 24 of 58 patients (41%) had AJCC stage I breast cancer; 19 of 58 patients (33%) had stage II disease; 8 of 58 patients (14%) had stage III disease; and 6 of 58 patients (10%) had stage IV disease (Table 4). Overall, 12 of 58 patients (21%) had detectable *STC-1* mRNA in their blood. The mean value for the 12 *STC-1*-positive blood specimens was 46,709 ECL U, whereas the mean value for the 48 *STC-1*-negative blood specimens was 6,394 ECLU. Three of 24 stage I patients (13%) had detectable *STC-1* mRNA in the blood, whereas 3 of 19 stage II patients (16%), 2 of 8 stage III patients (25%), and 4 of 6 stage IV patients (67%) were positive for *STC-1* mRNA.

***STC-1* Expression in Blood and Clinical Correlation.** Clinicopathological data were reviewed and analyzed for the 58 breast cancer patients who underwent RT-PCR analysis of their blood. Age, tumor grade, tumor type, Bloom-Richardson grading score, size of primary tumor, TNM stage, overall AJCC stage, number of involved axillary LNs, and estrogen/progesterone receptor status were evaluated relative to *STC-1* expression using χ^2 and Wilcoxon rank-sum tests in all 58 patients. The presence of *STC-1* mRNA in the blood significantly correlated with AJCC stage ($P = 0.03$, Wilcoxon rank-sum test), size of the primary tumor ($P < 0.03$, Wilcoxon rank-sum test), and the presence of distant metastases ($P = 0.003$, χ^2 test), all of which are established prognostic factors for breast cancer (Table 4). All other clinicopathological parameters failed to reach significance with respect to *STC-1* marker status. The number of involved axillary LNs approached significance ($P = 0.06$, Wilcoxon rank-sum test) with respect to RT-PCR status of the blood. Due to the small number of clinical disease-related events in this group of predominantly early-stage patients, correlation of *STC-1* blood marker status with disease recurrence and death was not feasible. Long-term follow-up will be required to further define the precise prognostic significance of detection of *STC-1* mRNA in the blood.

STC-1 ISH of Cell Lines and Primary Tumors. A chromogenic ISH assay was performed to confirm and localize the presence of *STC-1* mRNA in paraffin-embedded breast cancer primary tumors. The breast cancer cell lines BT-20 and MCF-7 and paraffin-embedded ILC and IDC tumors were assessed for *STC-1* mRNA expression using a *STC-1* antisense RNA probe. Both breast cancer cell lines (Fig. 2) and breast tumor cells (Figs. 3 and 4) were found to express *STC-1* mRNA. The *STC-1* ISH assay revealed dense and homogenous cytoplasmic staining in all breast cancer tumor cells, but no staining was seen in the surrounding nonductal mammary cells. Negative control ISH assays lacking the RNA probe did not stain breast cancer cell lines or primary tumors.

DISCUSSION

To date, the study of the mechanisms early breast cancer metastasis has been focused primarily on tumor cell spread to regional LNs. However, the presence of occult circulating metastatic tumor cells in the blood and BM may actually represent the earliest sign of systemic spread, and there is growing evidence that even this very early phase of systemic disease spread may be of prognostic importance. A significant correlation between prognosis and the detection of occult circulating breast tumor cells in the BM or peripheral blood has been confirmed in a number of studies to date (28–38). In terms of primary tumor characteristics, the detection of isolated tumor cells in the BM of breast cancer patients has been shown by other laboratories to correlate with increased primary tumor stage, increased nodal stage, the presence of vascular invasion, HER-2/neu overexpression, and estrogen/progesterone receptor expression (39). Although metastasis to the regional LNs has generally been thought to be the most significant prognostic factor with respect

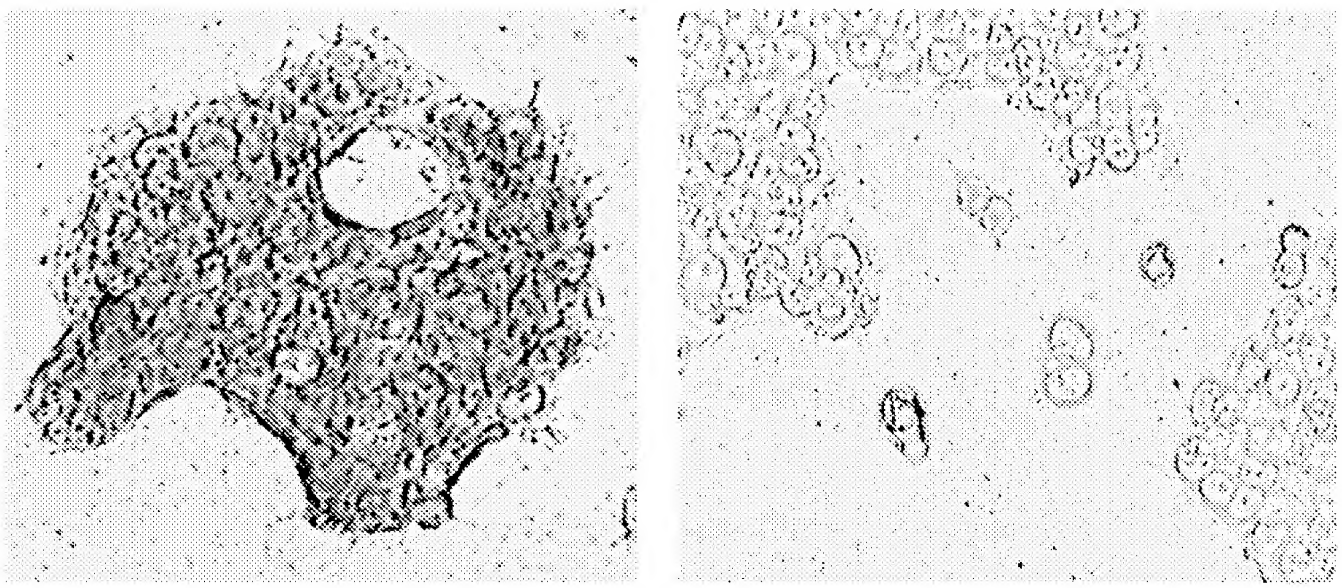


Fig. 2 Representative ISH of breast cancer cell line with *STC-1* antisense RNA probe. MCF-7 breast cancer cell line labeled with *STC-1* RNA probe (*left panel*) and negative control (no RNA; *right panel*).

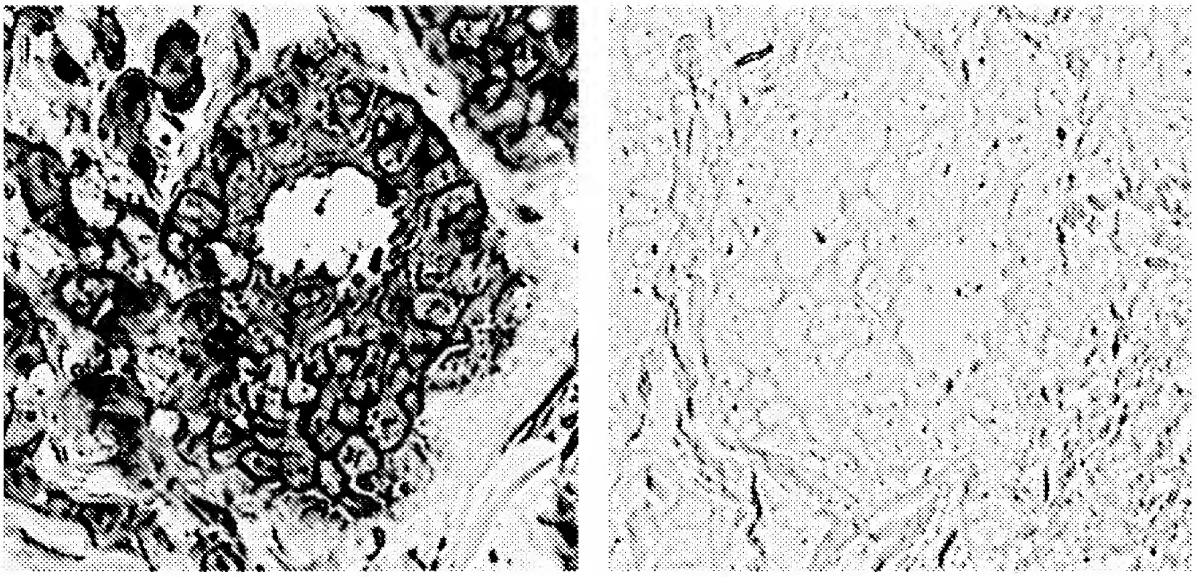


Fig. 3 Representative ISH of primary breast cancer tumor with *STC-1* antisense RNA probe. IDC labeled with *STC-1* RNA probe (*left panel*) and negative control (no RNA; *right panel*).

to predicting later recurrence and death (40), there is evidence that BM micrometastasis may be an even more significant prognostic factor than micrometastasis to the regional LNs (31, 41). Thus, the early detection of occult circulating tumor cells in the BM and blood may be more informative than the detection of early metastatic disease in the regional axillary LNs, including the sentinel LN(s).

The optimal detection of occult breast cancer tumor cells requires a sensitive assay, and one that is highly specific. The *STC-1* RT-PCR assay appears to fulfill these requirements when applied to the blood and BM of patients with breast cancer. Several clinically important applications of this assay can be considered. In addition to its potential value in ultrastaging patients without clinical evidence of systemic metastatic disease, another potentially important application of the *STC-1* RT-PCR assay might be to follow tumor progression and/or response to therapy when metastatic disease is not detectable by other means. Yet another potential application of the *STC-1* RT-PCR breast cancer assay is the evaluation of patients with suspected metastatic breast tumors that cannot be safely or

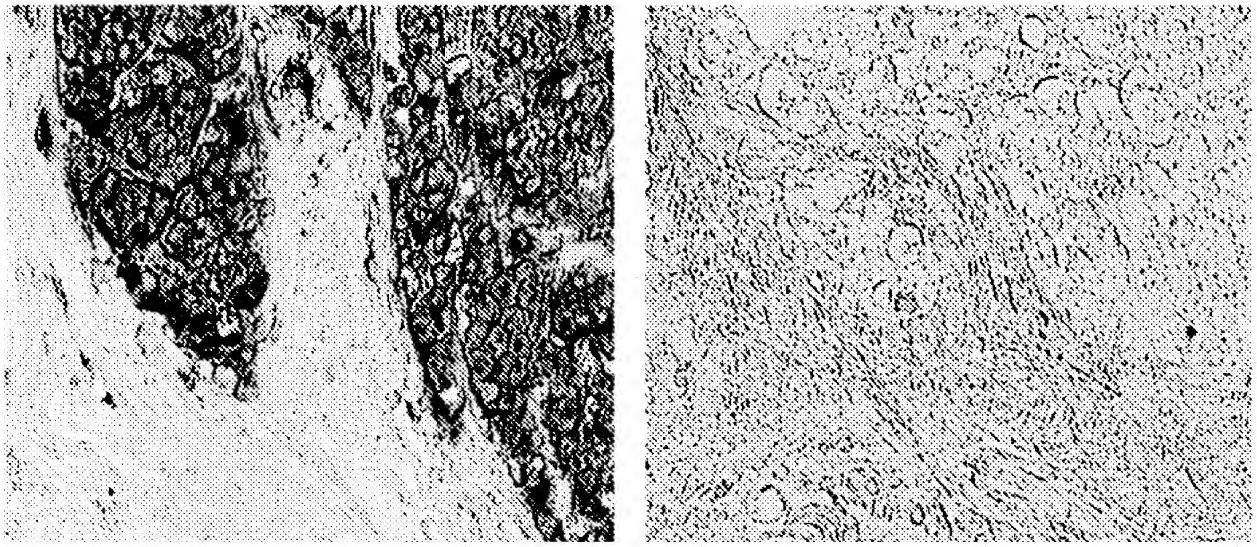


Fig. 4 Representative ISH of primary breast cancer tumor with *STC-1* antisense RNA probe. ILC labeled with *STC-1* RNA probe (*left panel*) and negative control (no RNA; *right panel*).

adequately biopsied by surgical means. The identification of subclinical metastatic breast cancer disease may also allow for the earlier application of adjuvant therapy, at a time when the patient's low level of tumor burden may result in the improved efficacy of such treatments in terms of disease progression and, possibly, survival. The early use of bisphosphonates or other osteoclast inhibitors in patients with occult BM disease might also significantly retard the development of clinically symptomatic skeletal metastases in patients without other clinical evidence of systemic disease. Such an assay could also prove useful to more accurately stage and stratify early-stage patients into clinical trials.

The *STC-1* RT-PCR assay appears to provide a highly sensitive method for detecting occult breast cancer tumor cells in the blood and BM and is therefore a promising candidate tumor marker for a single or multiple marker RT-PCR breast cancer molecular diagnostic assay. Moreover, in this study, the detection of circulating tumor cells in the BM and blood of patients with breast cancer by the *STC-1* RT-PCR assay was significantly correlated with known clinicopathological prognostic factors. Relapse of disease and death due to disease are prolonged in breast cancer relative to other tumor types, making it difficult to identify early correlation between the presence of occult circulating tumor cells and clinical outcomes such as disease-free survival and overall survival. However, the strong correlation in this study between the detection of occult circulating tumor cells and known clinicopathological prognostic factors further adds to the available evidence that subclinical disease in the blood, and particularly in the BM, is likely to be clinically important in breast cancer. Breast cancer has a well-known propensity to metastasize to the skeleton, subjecting patients to pain and fractures due to the resulting osteolytic lesions. Because skeletal metastases most likely arise from circulating tumor cells in the blood and BM, the ability to detect these clinically and radiographically invisible metastatic tumor cells may allow for the earlier application of adjuvant therapy, at a time when the patient metastatic tumor burden is still minimal. The adjuvant treatment armamentarium available for breast cancer patients rivals that of most other cancers. Thus, for patients with subclinical circulating tumor cells in the BM and/or blood as their only site(s) of disease, the early and aggressive application of hormonal, chemotherapeutic, or osteoclast inhibitor therapies may have an important role in slowing disease progression beyond that which is currently obtained by using these agents only after gross metastatic disease is detected. Indeed, the prognostic impact of circulating occult tumor cells and the effects of treating patients with such minimal disease deserve further study within a clinical trial setting.

Additional study of the physiological role of *STC-1* protein in both normal and cancer cells is also needed. In our study, considerable variability in *STC-1* mRNA copy levels was identified among different human breast cancer cell lines. Although the prognostic significance of *STC-1* gene expression/overexpression in breast cancer, if any, is unknown at this time, there is recent evidence of up-regulation of *STC-1* gene expression in human breast cancer cells subjected to hypoxic conditions *in vitro* (42). This finding suggests that *STC-1* might potentially confer a phenotypic resistance to the relatively hypoxic environment present within both tumors and bone.

In summary, we assessed *STC-1* as a potential molecular tumor marker for occult breast cancer in the BM and blood of patients with AJCC stage 0–IV breast cancer. *STC-1* mRNA, as detected by RT-PCR, appears to be highly sensitive and specific to occult breast cancer cells and is not expressed in normal blood and BM cells. Although few disease-related clinical events have occurred in the relatively early-stage patients evaluated in this study to date, the presence of breast cancer-associated *STC-1* mRNA in the BM and blood correlated significantly with the primary clinicopathological determinants of disease outcome (43). These findings, in addition to previous

studies demonstrating the prognostic significance of occult circulating breast tumor cells in the blood and BM, suggest that *STC-1* may be a useful and highly sensitive new molecular marker for breast cancer.

ACKNOWLEDGMENTS

We thank Dr. Y. Fujiwara (Osaka University, Osaka, Japan) and Dr. H. J. Wang (Department of Biomathematics, UCLA School of Medicine) for assistance with this study, and IGEN Int. Inc., for use of instrumentation.

REFERENCES

1. Giuliano, A. E., Kirgan, D. M., Guenther, J. M., and Morton, D. L. Lymphatic mapping and sentinel lymphadenectomy for breast cancer. Ann. Surg., *220:* 391–398, 1994.

2. Turner, R. R., Ollila, D. W., Krasne, D. L., and Giuliano, A. E. Histopathologic validation of the sentinel lymph node hypothesis for breast carcinoma. Ann. Surg., *226:* 271–276, 1997.

3. Hellman, S. Karnofsky Memorial Lecture: Natural history of small breast cancers. J. Clin. Oncol., *12:* 2229–2234, 1994.

4. Kamby, C., and Sengelov, L. Pattern of dissemination and survival following isolated locoregional recurrence of breast cancer. A prospective study with more than 10 years of follow up. Breast Cancer Res. Treat., *45:* 181–192, 1997.

5. Chang, A. C., Janosi, J., Hulsbeek, M., de Jong, D., Jeffrey, K. J., Noble, J. R., and Reddel, R. R. A novel human cDNA highly homologous to the fish hormone stanniocalcin. Mol. Cell. Endocrinol., *112:* 241–247, 1995.

6. Chang, A. C., Jeffrey, K. J., Tokutake, Y., Shimamoto, A., Neumann, A. A., Dunham, M. A., Cha, J., Sugawara, M., Furuichi, Y., and Reddel, R. R. Human stanniocalcin (STC): genomic structure, chromosomal localization, and the presence of CAG trinucleotide repeats. Genomics, *47:* 393–398, 1998.

7. Wagner, G. F., Jaworski, E. M., and Haddad, M. Stanniocalcin in the seawater salmon: structure, function, and regulation. Am. J. Physiol., *274:* R1177–R1185, 1998.

8. Hermsen, M. A., Baak, J. P., Meijer, G. A., Weiss, J. M., Walboomers, J. W., Snijders, P. J., and van Diest, P. J. Genetic analysis of 53 lymph node-negative breast carcinomas by CGH and relation to clinical, pathological, morphometric, and DNA cytometric prognostic factors. J. Pathol., *186:* 356–362, 1998.

9. Anbazhagan, R., Fujii, H., and Gabrielson, E. Allelic loss of chromosomal arm 8p in breast cancer progression. Am. J. Pathol., *152:* 815–819, 1998.

10. Sigbjornsdottir, B. I., Ragnarsson, G., Agnarsson, B. A., Huiping, C., Barkardottir, R. B., Egilsson, V., and Ingvarsson, S. Chromosome 8p alterations in sporadic and BRCA2 999del5 linked breast cancer. J. Med. Genet., *37:* 342–347, 2000.

11. Yokota, T., Yoshimoto, M., Akiyama, F., Sakamoto, G., Kasumi, F., Nakamura, Y., and Emi, M. Localization of a tumor suppressor gene associated with the progression of human breast carcinoma within a 1-cM interval of 8p22–p23.1. Cancer (Phila.), *85:* 447–452, 1999.

12. Varghese, R., Wong, C. K., Deol, H., Wagner, G. F., and DiMattia, G. E. Comparative analysis of mammalian stanniocalcin genes. Endocrinology, *139:* 4714–4725, 1998.

13. Wagner, G. F., Vozzolo, B. L., Jaworski, E., Haddad, M., Kline, R. L., Olsen, H. S., Rosen, C. A., Davidson, M. B., and Renfro, J. L. Human stanniocalcin inhibits renal phosphate excretion in the rat. J. Bone Miner. Res., *12:* 165–171, 1997.

14. Olsen, H. S., Cepeda, M. A., Zhang, Q. Q., Rosen, C. A., and Vozzolo, B. L. Human stanniocalcin: a possible hormonal regulator of mineral metabolism. Proc. Natl. Acad. Sci. USA, *93:* 1792–1796, 1996.

15. Madsen, K. L., Tavernini, M. M., Yachimec, C., Mendrick, D. L., Alfonso, P. J., Buergin, M., Olsen, H. S., Antonaccio, M. J., Thomson, A. B., and Fedorak, R. N. Stanniocalcin: a novel protein regulating calcium and phosphate transport across mammalian intestine. Am. J. Physiol., *274:* 96–102, 1998.

16. DiMattia, G. E., Varghese, R., and Wagner, G. F. Molecular cloning and characterization of stanniocalcin-related protein. Mol. Cell. Endocrinol., *146:* 137–140, 1998.

17. Wagner, G. F., Hampong, M., Park, C. M., and Copp, D. H. Purification, characterization, and bioassay of teleocalcin, a glycoprotein from salmon corpuscles of Stannius. Gen. Comp. Endocrinol., *63:* 481–491, 1986.

18. Slade, M. J., Smith, B. M., Sinnett, H. D., Cross, N. C., and Coombes, R. C. Quantitative polymerase chain reaction for the detection of micrometastases in patients with breast cancer. J. Clin. Oncol., *17:* 870–879, 1999.

19. Bostick, P. J., Chatterjee, S., Chi, D. D., Huynh, K. T., Giuliano, A. E., Cote, R., and Hoon, D. S. Limitations of specific reverse-transcriptase polymerase chain reaction markers in the detection of metastases in the lymph nodes and blood of breast cancer patients. J. Clin. Oncol., *16:* 2632–2640, 1998.

20. Brugger, W., Buhring, H. J., Grunebach, F., Vogel, W., Kaul, S., Muller, R., Brummendorf, T. H., Ziegler, B. L., Rappold, I., Brossart, P., Scheding, S., and Kanz, L. Expression of MUC-1 epitopes on normal bone marrow: implications for the detection of micrometastatic tumor cells. J. Clin. Oncol., *17:* 1535–1544, 1999.

21. Lambrechts, A. C., Bosma, A. J., Klaver, S. G., Top, B., Perebolte, L., van't Veer, L. J., and Rodenhuis, S. Comparison of immunocytochemistry, reverse transcriptase polymerase chain reaction, and nucleic acid sequence-based amplification for the detection of circulating breast cancer cells. Breast Cancer Res. Treat., *56:* 219–231, 1999.

22. Merrie, A. E., Yun, K., Gunn, J., Phillips, L. V., and McCall, J. L. Analysis of potential markers for detection of submicroscopic lymph node metastases in breast cancer. Br. J. Cancer, *80:* 2019–2024, 1999.

23. Hoon, D. S., Wang, Y., Dale, P. S., Conrad, A. J., Schmid, P., Garrison, D., Kuo, C., Foshag, L. J., Nizze, A. J., and Morton, D. L. Detection of occult melanoma cells in blood with a multiple-marker polymerase chain reaction assay. J. Clin. Oncol., *13:* 2109–2116, 1995.

24. Miyashiro, I., Kuo, C., Huynh, K., Iida, A., Morton, D., Bilchik, A., Giuliano, A., and Hoon, D. S. Molecular strategy for detecting metastatic cancers with use of multiple tumor-specific MAGE-A genes. Clin. Chem., *47:* 505–512, 2001.

25. O'Connell, C. D., Juhasz, A., Kuo, C., Reeder, D. J., and Hoon, D. S. Detection of tyrosinase mRNA in melanoma by reverse transcription-PCR and electrochemiluminescence. Clin. Chem., *44:* 1161–1169, 1998.

26. Bilchik, A., Miyashiro, M., Kelley, M., Kuo, C., Fujiwara, Y., Nakamori, S., Monden, M., and Hoon, D. S. Molecular detection of metastatic pancreatic carcinoma cells using a multimarker reverse transcriptase-polymerase chain reaction assay. Cancer (Phila.), *88:* 1037–1044, 2000.

27. Hoon, D. S., Sarantou, T., Doi, F., Chi, D. D., Kuo, C., Conrad, A. J., Schmid, P., Turner, R., and Guiliano, A. Detection of metastatic breast cancer by β-hCG polymerase chain reaction. Int. J. Cancer, *69:* 369–374, 1996.

28. Solomayer, E. F., Diel, I. J., Salanti, G., Hahn, M., Gollan, C., Schutz, F., and Bastert, G. Time independence of the prognostic impact of tumor cell detection in the bone marrow of primary breast cancer patients. Clin. Cancer Res., *7:* 4102–4108, 2001.

29. Ooka, M., Tamaki, Y., Sakita, I., Fujiwara, Y., Yamamoto, H., Miyake, Y., Sekimoto, M., Ohue, M., Sugita, Y., Miyoshi, Y., Ikeda, N., Noguchi, S., and Monden, M. Bone marrow micrometastases detected by RT-PCR for mammaglobin can be an alternative prognostic factor of breast cancer. Breast Cancer Res. Treat., *67:* 169–175, 2001.

30. Janni, W., Hepp, F., Rjosk, D., Kentenich, C., Strobl, B., Schindlbeck, C., Hantschmann, P., Sommer, H., Pantel, K., and Braun, S. The fate and prognostic value of occult metastatic cells in the bone marrow of patients with breast carcinoma between primary treatment and recurrence. Cancer (Phila.), *92:* 46–53, 2001.

31. Braun, S., Cevatli, B. S., Assemi, C., Janni, W., Kentenich, C. R., Schindlbeck, C., Rjosk, D., and Hepp, F. Comparative analysis of micrometastasis to the bone marrow and lymph nodes of node-negative breast cancer patients receiving no adjuvant therapy. J. Clin. Oncol., *19:* 1468–1475, 2001.

32. Gerber, B., Krause, A., Muller, H., Richter, D., Reimer, T., Makovitzky, J., Herrnring, C., Jeschke, U., Kundt, G., and Friese, K. Simultaneous immunohistochemical detection of tumor cells in lymph nodes and bone marrow aspirates in breast cancer and its correlation with other prognostic factors. J. Clin. Oncol., *19:* 960–971, 2001.

33. Braun, S., Pantel, K., Muller, P., Janni, W., Hepp, F., Kentenich, C. R., Gastroph, S., Wischnik, A., Dimpfl, T., Kindermann, G., Riethmuller, G., and Schlimok, G. Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. N. Engl. J. Med., *342:* 525–533, 2000.

34. Pantel, K., Cote, R. J., and Fodstad, O. Detection and clinical importance of micrometastatic disease. J. Natl. Cancer. Inst. (Bethesda), *91:* 1113–1124, 1999.

35. Zach, O., Kasparu, H., Krieger, O., Hehenwarter, W., Girschikofsky, M., and Lutz, D. Detection of circulating mammary carcinoma cells in the peripheral blood of breast cancer patients via a nested reverse transcriptase polymerase chain reaction assay for mammaglobin mRNA. J. Clin. Oncol., *17:* 2015–2019, 1999.

36. Smith, B. M., Slade, M. J., English, J., Graham, H., Luchtenborg, M., Sinnett, H. D., Cross, N. C., and Coombes, R. C. Response of circulating tumor cells to systemic therapy in patients with metastatic breast cancer: comparison of quantitative polymerase chain reaction and immunocytochemical techniques. J. Clin. Oncol., *18:* 1432–1439, 2000.

37. Taback, B., Chan, A. D., Kuo, C. T., Bostick, P. J., Wang, H. J., Giuliano, A. E., and Hoon, D. S. Detection of occult metastatic breast cancer cells in blood by a multimolecular marker assay: correlation with clinical stage of disease. Cancer Res., *61:* 8845–8850, 2001.

38. Mansi, J. L., Gogas, H., Bliss, J. M., Gazet, J. C., Berger, U., and Coombes, R. C. Outcome of primary-breast-cancer patients with micrometastases: a long-term follow-up study. Lancet, *354:* 197–202, 1999.

39. Naume, B., Borgen, E., Kvalheim, G., Karesen, R., Qvist, H., Sauer, T., Kumar, T., and Nesland, J. M. Detection of isolated tumor cells in bone marrow in early-stage breast carcinoma patients: comparison with preoperative clinical parameters and primary tumor characteristics. Clin. Cancer Res., *7:* 4122–4129, 2001.

40. Giuliano, A. E. Diseases of the breast. *In*: L. Way (ed.), Current Surgical Diagnosis and Treatment, 11th ed., pp. 319–343. New York: McGraw-Hill Companies, 2002.

41. Pantel, K., and Braun, S. Molecular determinants of occult metastatic tumor cells in bone marrow. Clin. Breast Cancer, *2:* 222–228, 2001.

42. Lal, A., Peters, H., St Croix, B., Haroon, Z. A., Dewhirst, M. W., Strausberg, R. L., Kaanders, J. H., van der Kogel, A. J., and Riggins, G. J. Transcriptional response to hypoxia in human tumors. J. Natl. Cancer Inst. (Bethesda), *93:* 1337–1343, 2001.

43. Fitzgibbons, P. L., Page, D. L., Weaver, D., Thor, A. D., Allred, D. C., Clark, G. M., Ruby, S. G., O'Malley, F., Simpson, J. F., Connolly, J. L., Hayes, D. F., Edge, S. B., Lichter, A., and Schnitt, S. J. Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999. Arch. Pathol. Lab. Med., *124:* 966–978, 2000.

0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(11):4951–4960

doi: 10.1210/en.2005-0365

Hypoxia-Inducible Factor-1-Mediated Activation of Stanniocalcin-1 in Human Cancer Cells

Ho Y. Yeung, Keng P. Lai, Hoi Y. Chan, Nai K. Mak, Graham F. Wagner, and Chris K. C. Wong

Department of Biology (H.Y.Y., K.P.L., H.Y.C., N.K.M., C.K.C.W.), Hong Kong Baptist University, Kowloon Tong, Hong Kong; and Departments of Physiology and Pharmacology (G.F.W.), The University of Western Ontario, London, Ontario, Canada N6A 5B8

Stanniocalcin-1 (STC1) is an endocrine hormone originally discovered in the corpuscles of Stannius, endocrine glands on kidneys of bony fishes, and also has been identified in mammals. The mammalian STC1 gene is widely expressed in various tissues and appears to be involved in diverse biological processes. There is growing evidence to suggest that altered patterns of gene expression have a role in human cancer development. Recently STC1 has been identified as a stimulator of mitochondrial respiration and has been hypothesized to be functionally related to the Warburg effect, of which hypoxia-inducible factor (HIF)-1 plays a key role in reprogramming tumor metabolism. This prompted us to examine the involvement of HIF-1 in the regulation of STC1 expression in tumor hypoxia. Our data reveal that hypoxia can stimulate STC1 gene expression in various human cancer cell lines, including those derived from colon carcinomas, nasopharyngeal cancer (CNE-2, HONE-1, HK-1), and ovarian cancer (CaOV3, OVCAR3, SKOV3). By far, the greatest response was observed in CNE-2 cells. In further studies on CNE-2 cells, desferrioxamine, cobalt chloride, and O_2 depletion all increased HIF-1α protein and STC1 mRNA levels. Desferrioxamine treatment, when coupled with Fe replenishment, abolished these effects. RNA interference studies further confirmed that endogenous HIF-1α was a key factor in hypoxia-induced STC1 expression. The ability of vascular endothelial growth factor to stimulate STC1 expression in CNE-2 cells was comparatively low. Collectively, the present findings provide the first evidence of HIF-1 regulation of STC1 expression in human cancer cells. The studies have implications as to the role of STC1 in hypoxia induced adaptive responses in tumor cells. (*Endocrinology* 146: 4951–4960, 2005)

STANNIOCALCIN-1 (STC1) IS an endocrine hormone originally discovered in the corpuscles of Stannius, endocrine glands on kidneys of bony fishes (1), and has also been identified in mammals. Human STC1 is encoded by a single copy gene localized on chromosome 8p11.2-p21 (2). The gene comprises four exons that encode 247 amino acids with 11 cysteine residues (2, 3). Numerous studies have shown that the mammalian STC1 is expressed in various tissues such as heart, lung, liver, adrenal, kidney, prostate, and ovary (3–5). The gene is modulated in numerous developmental, physiological and pathological processes, including cancer, pregnancy, lactation, angiogenesis, organogenesis, cerebral ischemia, and hypertonic stress (6, 7). Furthermore, STC1 overexpression in transgenic mouse models results in high serum phosphate, dwarfism, increased vascular density, mitochondrial hypertrophy, and increased rates of respiration (8, 9). The STC1 receptor has not been cloned. However, STC1 binding sites have been identified on kidney and liver cell membranes and the outer and inner mitochondrial membranes of both organs (10).

First Published Online August 18, 2005

Abbreviations: Act D, Actinomycin D; $CoCl_2$, cobalt chloride; DFX, desferrioxamine mesylate; HIF, hypoxia-inducible factor; Ndrg1, N-myc downstream-regulated gene 1; NO, nitric oxide; si, small interfering; SNP, sodium nitroprusside; STC1, stanniocalcin-1; VEGF, vascular endothelial growth factor.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Numerous lines of evidence show that the short arm of chromosome 8 includes at least two tumor suppressor genes (prostate, bladder, and colorectal carcinomas) and an amplified region associated with breast carcinoma (2, 11–15). Intriguingly the mammalian STC1 was cloned in a screen for cancer-related genes (4). There is also growing evidence that altered STC1 expression patterns may have a role in human cancer (6). Enhanced STC1 gene expression has been found in hepatocellular, colorectal, and breast carcinomas and medullary thyroid cancers (16–20). In contrast, a down-regulation of STC1 expression was found in breast and ovarian cancer cell lines (21–25). STC1 expression has been found to be induced by the RET- multiple endocrine neoplasia (MEN) 2B mutant protein, BRCA1 (a tumor suppressor gene that has an important role in breast and ovarian cancers), and vascular endothelial growth factor (VEGF) (19, 21, 23, 26–28). Some evidence has linked STC1 expression to the formation of tumor vasculature (17), and the possible use of STC1 expression levels for the diagnosis of human breast, hepatocellular, and colorectal cancers has been postulated (16, 20, 29). Although a considerable number of reports have indicated that STC1 is differentially expressed in tumors, compared with surrounding normal tissue, the pathological and biological significances of these observations require more investigation.

Despite the growing body of knowledge, little is known about STC1 signaling and its functions in cancer progression. In this report, we examined the regulation of STC1 gene expression in the human nasopharyngeal cancer cell line, CNE-2, and we demonstrated that the transcriptional factor,

hypoxia-inducible factor (HIF)-1, mediates the activation of STC1 expression in cells exposed to hypoxic stress.

Materials and Methods

Effects of hypoxia [desferrioxamine mesylate (DFX), cobalt chloride ($CoCl_2$) or O_2 depletion] on HIF-1α, STC1, and N-myc downstream-regulated gene 1 (Ndrg1) mRNA levels

The human nasopharyngeal carcinoma cell lines (*i.e.* CNE-2, HK-1, HONE-1), human ovarian cancer cell lines (*i.e.* CaOV3, OVCAR3, SKOV3), and human colorectal adenocarcinoma cell lines were maintained in their corresponding media and exposed to 50 μM DFX (Sigma, St. Louis, MO) treatment for 24 h. Total RNA was extracted and reverse transcribed, and STC1 mRNA levels were measured by real-time PCR. Significant inductions of STC1 mRNA were observed in the all cell lines. Among those, CNE-2 produced the most striking response to the treatment. Therefore, CNE-2 cells were used in all subsequent experiments to study the regulation of STC1 gene expression.

CNE-2 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) at a density of 2.5×10^4 cells/well in 12-well plates (Nunc, Nalge Nunc, Rochester, NY). The cells were incubated in 5% CO_2 at 37 C. After overnight incubation, the cells were exposed for 24–48 h to one of the following treatments: 1) 25, 50, or 100 μM DFX; 2) 250 μM $CoCl_2$; or 3) O_2 depletion. To achieve conditions of O_2 depletion, the cultures were maintained in an air-tight modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA) infused with a preanalyzed gas mixture (5% CO_2-95% N_2) at a flow rate of 25 liters/min for 5 min twice a day. The pO_2 was measured by a gas analyzer mounted with an O_2 sensor (Quest Technologies, Oconomowoc, WI). The O_2 content in the incubator chamber was maintained in a range of 1–3% throughout the incubation.

Effects of sodium nitroprusside (SNP), $FeCl_3$, potassium ferrocyanide [$K_3Fe(CN)_6$], and VEGF on DFX-induced HIF-1α and STC mRNA levels

CNE-2 cells were exposed for 24 h to one of the following treatments: 1) 50 μM DFX; 2) 1000 μM SNP (Calbiochem, La Jolla, CA); 3) 500 μM $K_3Fe(CN)_6$; 4) 300 μM $FeCl_3$; or 5) 25–50 ng/ml VEGF (Upstate Biotechnology, Lake Placid, NY). In addition, DFX-treated CNE-2 cells were coexposed for 24 h to 100-1000 μM SNP, 100–500 μM $K_3Fe(CN)_6$, 300 μM $FeCl_3$, or 25–50 ng/ml VEGF. Total RNA and cell lysates were collected for real-time PCR and Western blot, respectively.

RNA extraction, PCR product verification, and real-time PCR

Cells were dissolved in TRIZOL reagent (Gibco/BRL, Gaithersburg, MD). Total RNA was extracted according to the manufacturer's instructions. The RNA A_{260}/A_{280} ratios were between 1.6 and 1.8. The primers were designed on the basis of the published sequence of human STC1 (CACACCCACGAGCTGACTTC-forward and TCTCCCTGGTTATGCACTCTCA-reverse), HIF-1α (TCCAGCAGACTCAAATACAAGAAC-forward and GTATGTGGGTAGGAGATGGAGATG-reverse), VEGF (CGAAACCATGAACTTTCTGC-forward and CCTCAGTGGGC ACACACTCC-reverse), Ndrg1 (GCCTTGTCCTTATCAACGTGAAC-forward and CTTGGG TCCATCCTGAGATCTTG-reverse), p21 (CTGCCGAAGTCAGTTCCTTGTG-forward and CATCCCCAGCCGGTTCTGAC-reverse), GADD45 (AGTGAGTGCAGAAAGCAGGC-forward and GCTGACTCAGGGCTTTGCTG), and actin (GACTACCTCATGAAGATCCTCACC-forward and TCTCCTTAATGTCACGCACGATT-reverse). The PCR was run for 30–35 cycles with a 56 C annealing cycle (1 min), a 72 C extension cycle (1 min), and a 95 C denaturing cycle (50 sec) plus final incubation at 72 C for 5 min. The PCR products (~140 bp for STC1, ~138 bp for HIF-1α, ~331 bp for VEGF, ~81 bp for Ndrg1, ~81 bp for p21, ~192 bp for GADD45, and ~85 bp for actin) were purified, subcloned into pCR II-TOPO (Invitrogen, Carlsbad, CA), and subjected to verification using an automated DNA sequencer (ABI 3700; PE Applied Biosystems, Foster City, CA).

Real-time PCR was conducted and the housekeeping gene, actin, was used as an internal standard. The treated cells were dissolved in TRIZOL reagent (Gibco/BRL), and total RNA was extracted as described above. Briefly, cDNA was synthesized from 1 μg of total cellular RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitated standards (10^4 to 10^8) and sample cDNAs were analyzed with the iCycler iQ real-time PCR detection system using iQ SYBR Green Supermix (Bio-Rad). The copy number for each sample was calculated and all the data were normalized to actin. The PCR conditions were 95 C for 3 min and 40 cycles of 95 C for 30 sec, 56 C for 30 sec, and 72 C for 1 min. Fluorescent signals were captured at 82 C, and the occurrence of primer-dimers and secondary products were inspected using melting-curve analysis. Control amplifications were done without either reverse transcription or RNA. After PCR amplification, the reaction products were resolved at 100 V on a 1% agarose gel with 0.5 μg/ml ethidium bromide. All glass- and plasticware were treated with diethyl pyrocarbonate and autoclaved.

Western blot analysis

The treated cells were washed with two to three changes of cold PBS. Adherent cells were scraped from the plastic surface and transferred to a microcentrifuge tube. The cells were pelleted and resuspended in a cold lysis buffer containing 250 mM Tris/HCl (pH 8.0), 1% Nonidet P-40, and 150 mM NaCl. After a 10-min incubation on ice, the lysed cells were pelleted and supernatants assayed for protein concentration (DC Protein Assay Kit II, Bio-Rad Pacific Ltd., Kowloon, Hong Kong). Samples were subjected to electrophoresis in NuPage 4–12% Bis-Tris gradient gels (Invitrogen). Gels were blotted onto polyvinyl difluoride membrane (PerkinElmer Life Sciences, Norwalk, CT). Western blot was conducted using rabbit antibodies to STC1 (9) or HIF1α (BD Transduction Laboratories, San Diego, CA), followed by incubation with horseradish peroxidase-conjugated goat antirabbit antibody. Specific bands were visualized with chemiluminescent reagent (Western-Lightening Plus, PerkinElmer Life Sciences). Blots were then washed in PBS and reprobed with rabbit antiactin serum (Sigma).

RNA Interference. One day before transfection, CNE-2 cells were plated into 6-well plates. The cells were grown to 70% confluence and then mock transfected or transfected with 20 nM of siCONTROL nontargeting small interfering (si)RNA duplex, human HIF-1α-specific siRNA duplex, or human VEGF-specific siRNA duplex (Dharmacon, Lafayette, CO) using siLectFect according to the manufacturer's instructions (Bio-Rad). The cells were then exposed to 50 μM DFX. Additional HIF-1α RNA interference experiments were conducted in the air-tight modular incubator chamber (O_2 content 1–3%). All treatments were carried out in triplicate. Total RNA and cell lysates were collected for real-time PCR and Western blot, respectively, to determine expression levels of HIF-1α, STC1, and Ndrg1.

Northern blot analysis

Untreated CNE-2 cells or DFX-treated cells transfected with either siCONTROL nontargeting siRNA or human HIF-1α-specific siRNA were harvested. Total RNA was isolated as outlined above. Twenty micrograms of total RNA per lane were resolved on 1% agarose/formaldehyde gels and subjected to Northern blot analysis using random-primed, ^{32}P-labeled human STC1 or actin cDNA probes (1×10^6 cpm/ml) in Rapid-Hyb buffer (Amersham Biosciences, Piscataway, NJ) at 65 C for 2 h. To reduce nonspecific binding, all the blots were washed twice in 2× saline sodium citrate containing 0.1% sodium dodecyl sulfate for 20 min at room temperature, followed by 0.1× saline sodium citrate and 0.1% sodium dodecyl sulfate twice for 15 min at 65 C. Signal was then detected using x-ray film.

Statistical analysis

Drugs treatments were performed in triplicate in the same experiments, and individual experiments were repeated at least three times. All data are represented as the mean ± SEM. Statistical significance was assessed with a Student's *t* test or one-way ANOVA followed by Duncan's multiple range test. Groups were considered significantly different if $P < 0.05$.

Results

STC1 mRNA induction in human cancer cell lines under hypoxia treatments

Real-time PCR analysis of relative basal levels of STC1 mRNA in various human cancer cell lines are shown in Fig. 1A. Upon DFX treatment, STC1 transcript levels increased in all cancer cell lines, although with different levels of induction (Fig. 1B). Because the highest response was seen in CNE-2 cells, we selected this line for all subsequent studies. Figure 1C demonstrates the dose-dependent induction of STC1 mRNA by DFX treatment for 24 h. Induction also occurred after 24 h of O_2 depletion or $CoCl_2$ treatment (Fig. 1D). Figure 1E points to the possible involvement of HIF-1α in these responses because all the above treatments inhibited the activity of HIF prolyl hydroxylases (30) and in doing so the degradation of HIF-1α. To correlate the expression profiles of STC1 and HIF-1α, Fig. 2A demonstrates the induction of STC1 and HIF-1α protein by DFX at different time increments over 24 and/or 48 h. In contrast, HIF-1α mRNA levels remained

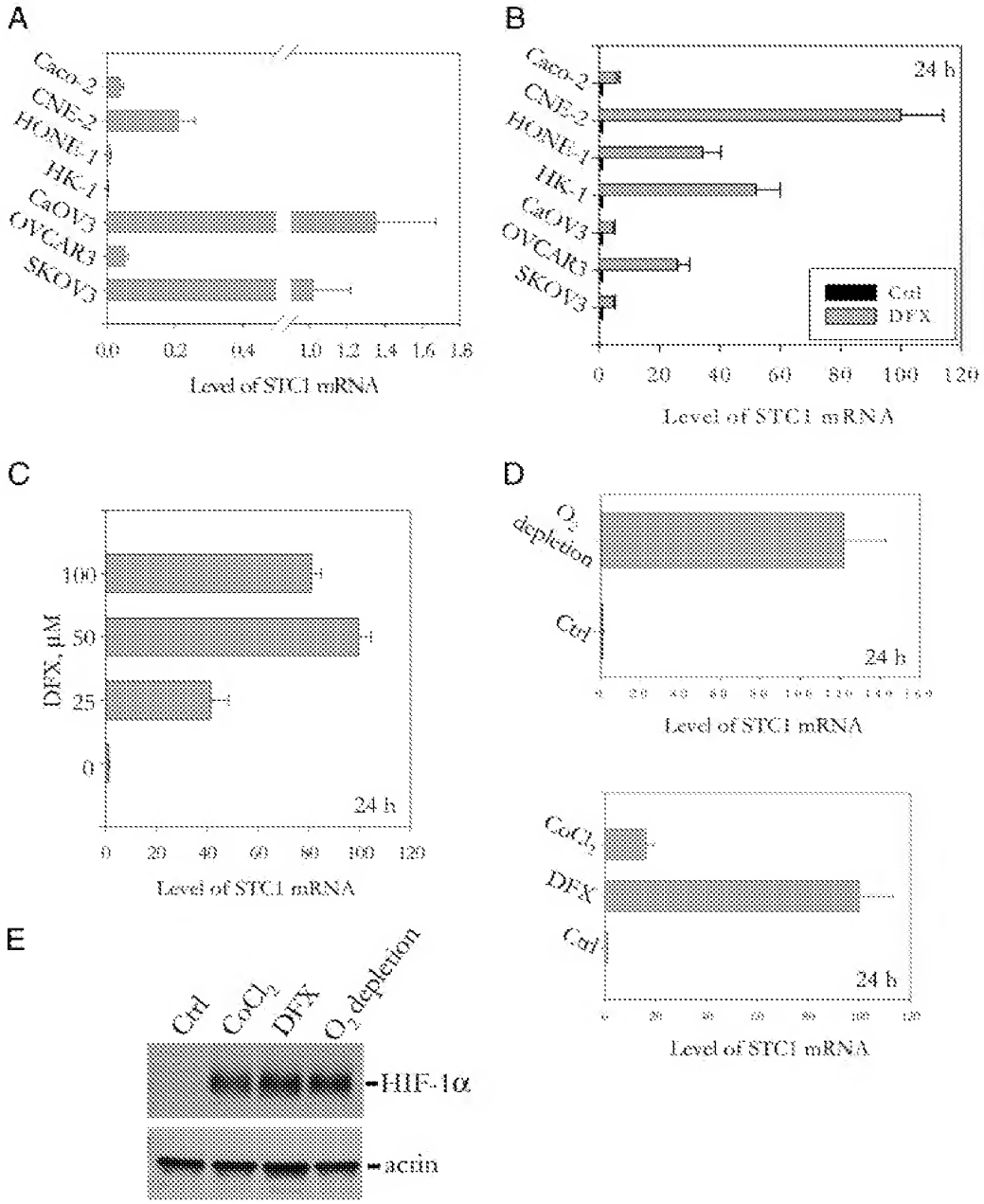


FIG. 1. Induction of STC1 mRNA and HIF-1α expressions in hypoxia-treated human cancer cells. A, Relative basal levels of STC1 mRNA in various types of human cancer cells. B, Induction of STC1 mRNA on DFX treatment (50 μM) for 24 h. The relative induction levels in various cell lines are shown in reference to their respective basal levels. STC1 mRNA levels were determined by real-time PCR and normalized to actin (five samples per point). C, Dose-dependent induction of STC1 mRNA expression in CNE-2 cells after 24 h of DFX treatment (25–100 μM). D, Induction of STC1 mRNA in CNE-2 cells 24 h after O_2 depletion (1–3%) or 250 μM $CoCl_2$ treatment. E, Western blot analysis of HIF-1α after 24 h of hypoxia in CNE-2 cells. Ctrl, Control.

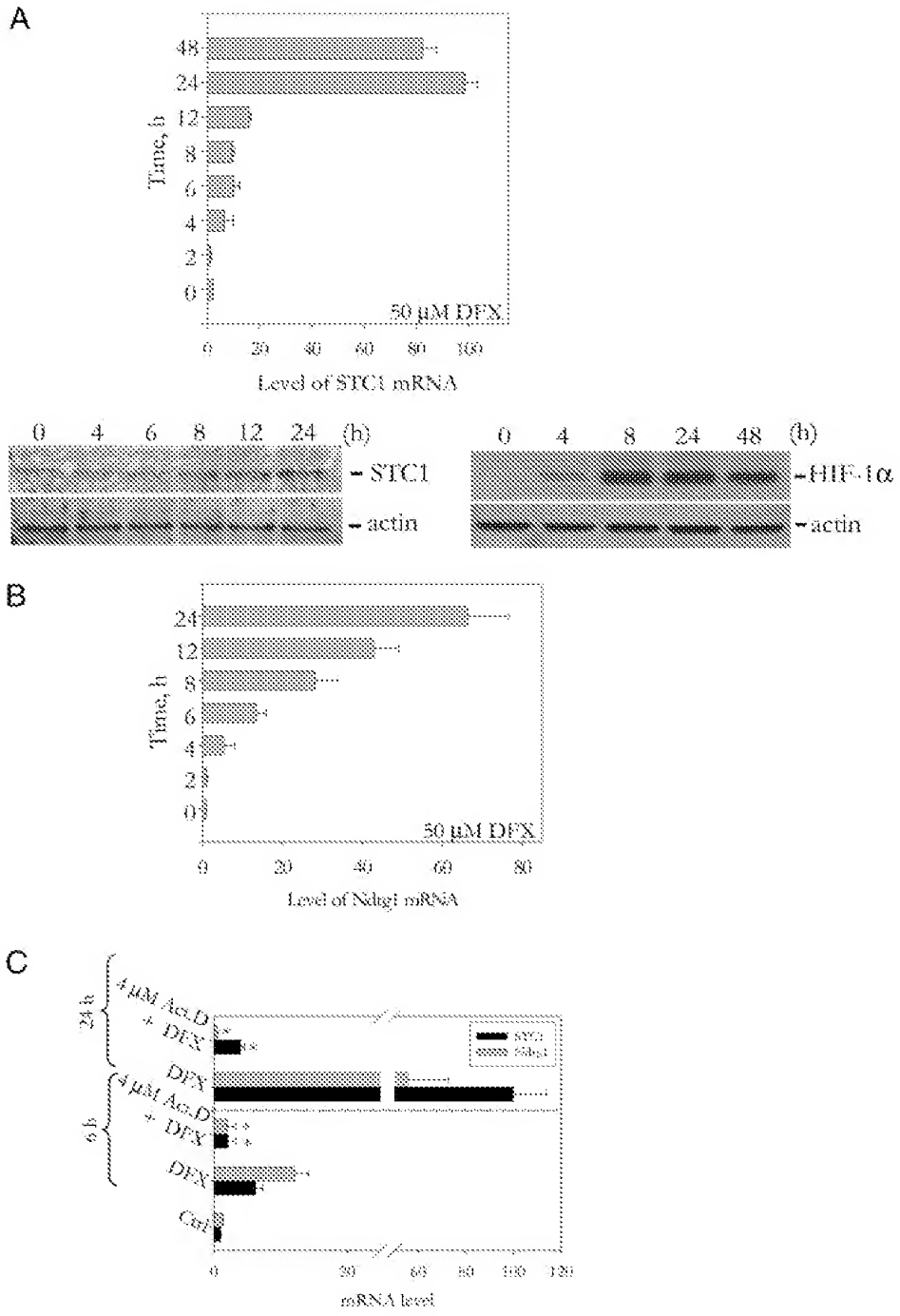


FIG. 2. Time-course inductions of STC1 and Ndrg1 mRNA and HIF-1α protein in DFX-treated CNE-2 cells. DFX (50 μM) caused a temporal induction of STC1 mRNA (A) and Ndrg1 mRNA levels (B), the effect peaking at 24–48 h. *Inset* between panels A and B, Cellular levels of STC1 and HIF-1α protein were also temporally induced by DFX treatment, as determined by Western blot. C, The stimulatory effects of DFX on both transcripts were significantly reduced by Act D. *, $P < 0.01$, compared with DFX treatment alone. Ctrl, Control.

constant (data not shown). A marked discrepancy was evident between the induction in STC1 mRNA levels (high) and STC1 protein levels (low). This was possibly attributable to the hypoxic stress, which can inhibit translation (31, 32). Hence, a reduction of STC1 mRNA translation efficiency may have occurred in our model. The fact that HIF-1α mRNA levels remained stable was in agreement with other reports indicating that HIF-1α is mainly regulated posttranslationally, not at the RNA level (33–35). We also analyzed Ndrg1 mRNA levels in CNE-2 cells throughout the DFX studies (Fig. 2B). The induction of Ndrg1 mRNA can serve as a positive control for the DFX treatment because the gene is known to be involved in cellular Fe metabolism and can be induced by HIF-1α (36). In this study, a significant induction in Ndrg1 gene expression was also observed in $CoCl_2$ or O_2 depletion-treated cells (data not shown).

It has been suggested that DFX treatment and/or Fe chelation may cause DNA damage (37, 38). This was considered as a possible cause of STC1 mRNA induction in the cells in this study. Moreover, the up-regulation of p21 and GADD45 mRNA are well-described responses to DNA damage and so can serve as good positive controls (36, 39). To assess this possibility, we measured the p21 and

GADD45 responses in CNE-2 cells after DFX treatment. Our results indicated, however, that there was no induction of p21 or GADD45 transcripts in DFX-treated cells (data not shown). Hence, in this study, DFX stimulation of STC1 gene expression does not appear to be related to DNA damage. To determine whether STC1 is transcriptionally regulated by hypoxia treatment, we incubated the cells with medium containing DFX and 4 μM actinomycin D (Act D). After 6 and 24 h of incubation, DFX mediated STC1 mRNA induction was almost completely blocked by Act D (Fig. 2C). Similar results were found for Ndrg1, which is also transcriptionally regulated by DFX (36).

The increase in STC1 expression after DFX treatment is HIF-1α dependent and can be reversed by SNP, $K_3Fe(CN)_6$, and iron (III) salts

From the data, it appeared likely that HIF-1α was directly involved in the regulation of STC1 expression. To test this hypothesis, we measured the levels of STC1 mRNA in DFX-treated cells in which HIF-1α activities were suppressed by chemical treatment. SNP [a nitric oxide (NO) donor], $K_3Fe(CN)_6$ as well as Fe (III) salts were used to reduce HIF-1α protein levels in DFX-treated cells. This approach can elucidate the role of HIF-1α in STC1 mRNA induction. SNP is known to inhibit HIF-1α (40) and was found to completely

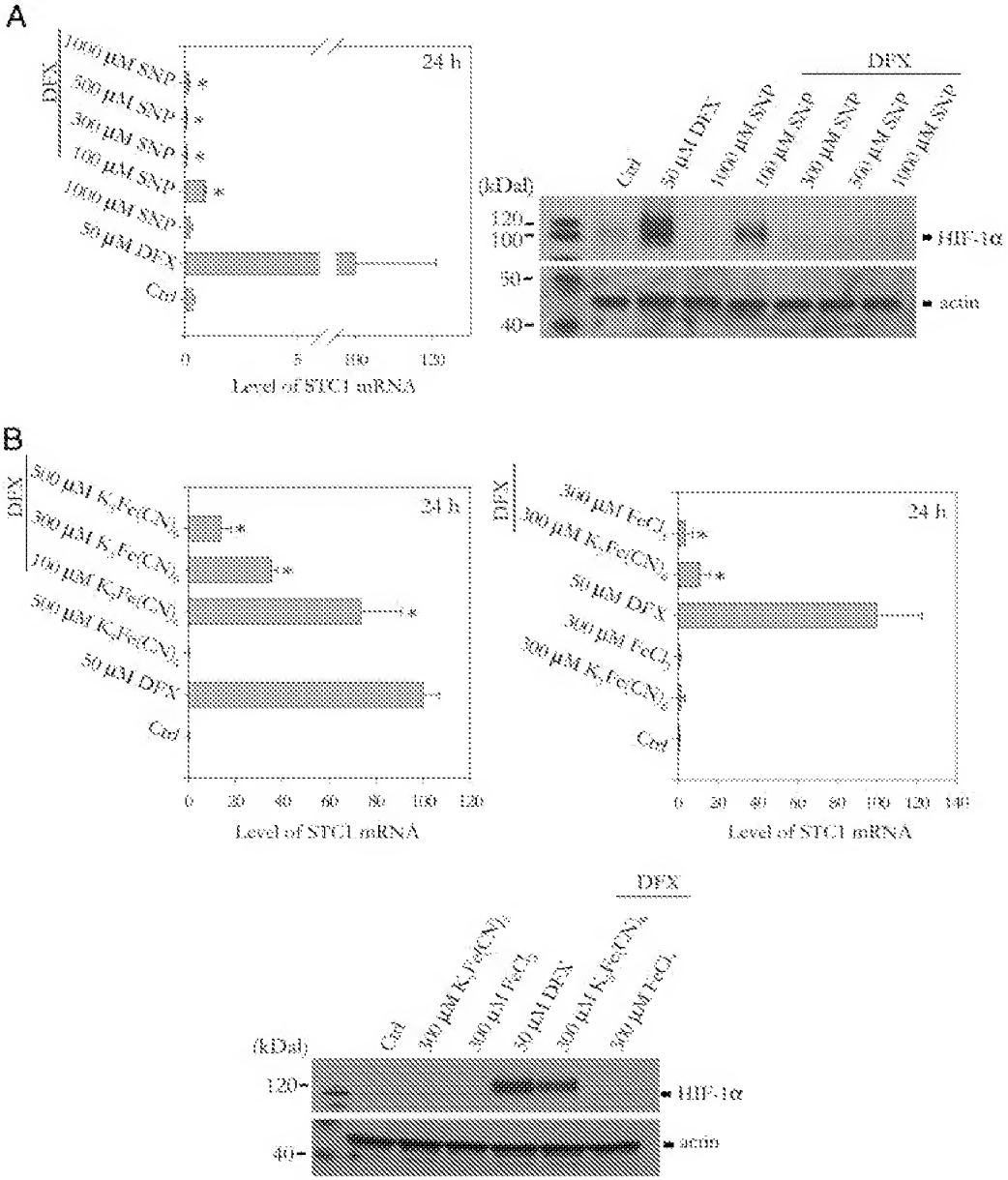


FIG. 3. Effect of SNP and Fe replenishment on DFX-induced STC1 and Ndrg1 mRNA and HIF-1α protein levels. DFX (50 μM)-induced CNE-2 cells were cotreated with SNP (100–1000 μM) (A) or $K_3Fe(CN)_6$ (100–500 μM) or 300 μM of $FeCl_3$ for 24 h (B). STC1 mRNA levels were measured by real-time PCR, whereas HIF-1α protein (*bottom panel*) was determined by Western blot. *, $P < 0.05$, compared with the DFX treatment alone. Ctrl, Control.

reduce the levels of HIF-1α protein as well as STC1 mRNA levels (Fig. 3A). $K_3Fe(CN)_6$, a compound structurally related to SNP but without any NO group, also decreased the levels of HIF-1α protein and significantly reduced STC1 mRNA levels (Fig. 3B). The Fe (III) salts abolished the effect of DFX on both HIF-1α and STC1 induction. The decrease of HIF-1α protein levels in SNP, $K_3Fe(CN)_6$, or Fe (III) salts treatment correlated well with the reductions in STC1 mRNA levels.

To determine whether STC1 induction was attributable to mitochondrial dysfunction, possibly resulting from the Fe-chelating effect of DFX, we conducted experiments to inhibit mitochondrial function in the cells. Psychosine (inhibitor of cytochrome *c* oxidase), rotenone [inhibitor of complex I-nicotinamide adenine dinucleotide, reduced form (NADH)-CoQ reductase], and 2-thenoyltrifluoroacetone (an inhibitor of electron complex II; Calbiochem) were used to treat the cells for 3–24 h. In this study, cellular ATP levels of all treated cells were significantly reduced, compared with controls. However, no increases in STC1 mRNA levels were observed (data not shown). Taken together, these results indicated that the induction of STC1 was highly correlated to HIF-1α protein levels and not to mitochondrial dysfunction.

STC1 induction is abolished in siRNA$_{HIF\text{-}1\alpha}$ transfected DFX-induced or O_2 depletion-treated cells

RNA interference assay was conducted to further verify the role of endogenous HIF-1α in hypoxia-induced STC1 gene expression. An siRNA$_{HIF\text{-}1\alpha}$ that specifically targets human HIF-1α mRNA was added to DFX-treated cells. The inhibitory effect of this siRNA$_{HIF\text{-}1\alpha}$ on HIF-1α expression was confirmed by both real-time PCR and Western blot, which showed the loss of transcript and protein, respectively (Fig. 4A). As shown in Fig. 4B, DFX-treated cells expressed high level of STC1 mRNA. This activation was almost completely abrogated by the treatment with siRNA$_{HIF\text{-}1\alpha}$ but not

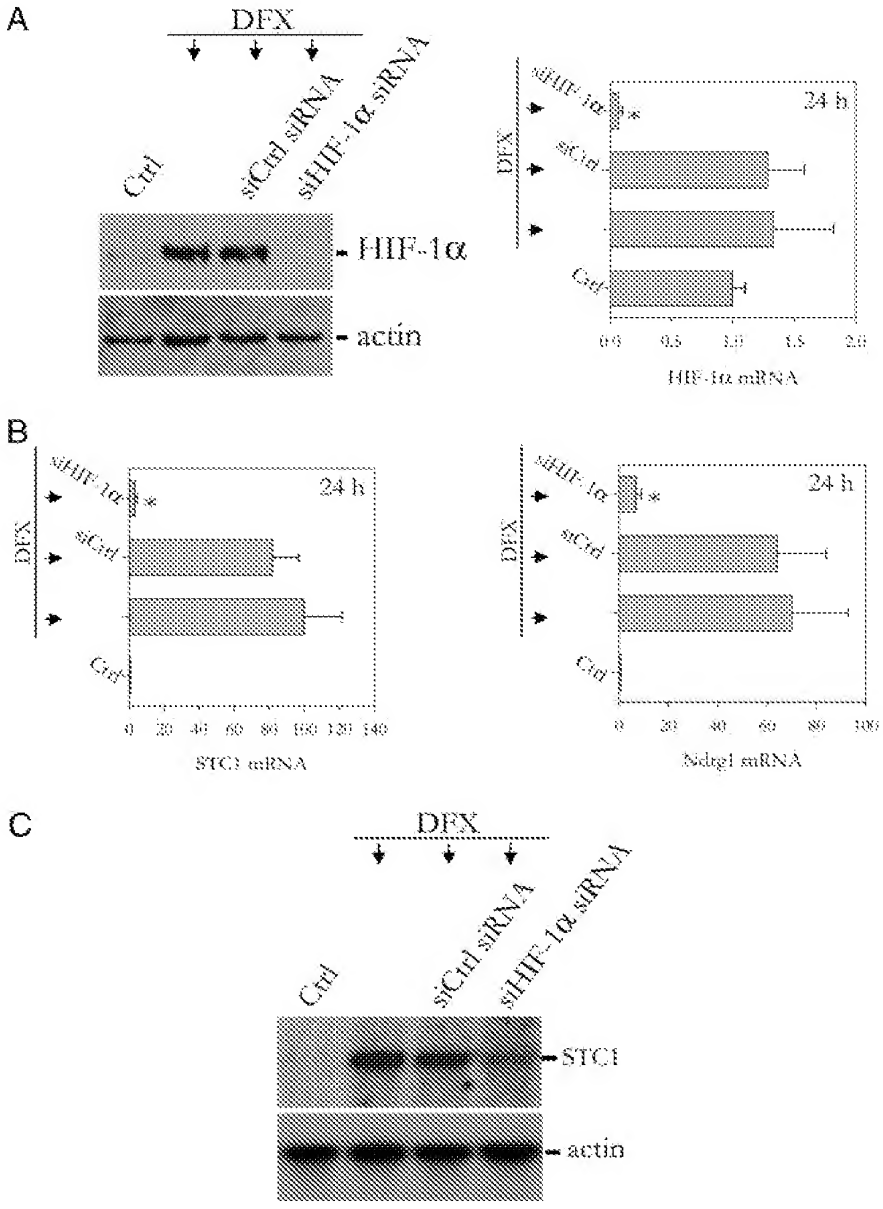


FIG. 4. Human HIF-1α RNA interference assay. DFX (50 μM)-induced CNE-2 cells were transfected with 20 nM of siCONTROL nontargeting siRNA duplex or human HIF-1α-specific siRNA duplex using siLectFect. Twenty-four hours after transfection, HIF-1α protein (*left*) and mRNA levels (*right*) (A) were determined by Western blot and real-time PCR. B, STC1 and Ndrg1 mRNA levels were measured by real-time PCR. DFX-induced HIF-1α, STC1, and Ndrg1 expression patterns were significantly abrogated by the treatment with siRNA-$_{HIF\text{-}1\alpha}$ but not in siCONTROL-transfected cells. *, $P < 0.01$, compared with the DFX treatment alone. C, Northern analysis of STC1 expression after RNA interference. Total RNA (20 μg/lane) was resolved on formaldehyde-1% agarose denaturing gels, transferred, and hybridized to a human STC1 cDNA probe. The blot was reprobed with an actin cDNA to normalize the STC1 signal in each lane. Results shown are from three or more independent experiments. Ctrl, Control.

by the mock-transfected or siCONTROL-transfected cells. The observation was further confirmed by Northern blot analysis (Fig. 4C). The expression of Ndrg1, a gene that is known to be regulated by HIF-1α, was also inhibited by $siRNA_{HIF\text{-}1\alpha}$ treatment. Reductions in HIF-1α and STC1 expression were also observed in $siRNA_{HIF\text{-}1\alpha}$-treated, O_2-depleted cells (data not shown). To exclude any possible off-target effects of the $siRNA_{HIF\text{-}1\alpha}$ treatment, we repeated the experiments with four individual $siRNA_{HIF\text{-}1\alpha}$ duplexes (5′-CAAGUCUAAAUCUGUGUCCUU-3′, 5′-GGACACAGAUUUAGACUUGUU-3′; 5′-UUUGUCUAGUGCUUCCAUCUU-3′, 5′-GAUGGAAGCACUAGACAAAUU-3′; 5′-CAAAGCGACAGAUAACACGUU-3′, 5′-CGUGUUAUCUGUCGCUUUGUU-3′; 5′-UCUGAUUCAACUUUGGUGAUU-3′, 5′-UCACCAAAGUUGAAUCAGAUU-3′) (Dharmacon). Similar suppressive effects on HIF-1α, Ndrg1, and STC1 mRNA levels were observed (data not shown). These results confirm that endogenous HIF-1α is involved in hypoxia-induced STC1 gene expression.

VEGF is also known to be transcriptionally stimulated by HIF-1. Therefore, in view of numerous reports demonstrating the stimulatory effects of VEGF on STC1 expression in human endothelial cells (23, 26–28), we decided to investigate the significance of VEGF to STC1 stimulation. In DFX-treated CNE-2 cells, 3- to 4-fold inductions of VEGF transcript levels were detected (Fig. 5A). Increasing doses of VEGF (25–50 ng/ml) stimulated STC1 gene expression (Fig. 5B). The magnitude of stimulation, however, was far below that induced by DFX treatment. To assess the possible role of endogenous VEGF in DFX-induced STC1 gene expression, RNA interference was conducted. An $siRNA_{VEGF}$ that specifically targets human VEGF mRNA was added to DFX-treated cells. The inhibitory effect of this $siRNA_{VEGF}$ on VEGF expression was confirmed by real-time PCR (Fig. 5C). However, DFX-induced expression of STC1 mRNA and HIF-1α were not affected by $siRNA_{VEGF}$ treatment. Additional experiments on CNE-2 cells cotreated with DFX (50 μM) and VEGF (25–50 ng/ml) were conducted. However, there was no significant difference in STC1 mRNA levels between DFX-treated and DFX/VEGF-treated cells (data not shown). These results further support the notion that endogenous HIF-1α is the key factor stimulating STC1 gene expression.

Discussion

Mammalian STC1 was cloned in the investigation of cancer-related genes (4) and independently by random sequencing of a fetal lung cDNA library (5). The gene is widely expressed in tissues as diverse as heart, lung, liver, adrenal, kidney, prostate, and ovary (3–5). More recently there has been increasing evidence to support the role of STC1 in human cancer development. The STC1 receptor has not been cloned; however, high-affinity binding sites have been identified on the outer and inner mitochondrial membranes in which STC-1 has stimulatory effects on electron transport (10). The evidence has put STC1 on an exclusive list of regulatory factors including NO, thyroid hormone, and TGFβ1, which can modulate mitochondrial functions (41–43). In view of its possible role in mitochondrial function, it has been hypothesized that STC1 expression may be related to the Warburg effect (6), in which HIF-1 plays a key role in modulating the expression of glycolytic enzymes and reprogramming of tumor metabolism (44, 45). HIF-1 is a key regulator in the cellular responses to oxygen deprivation and is well known to be involved in cancer progression (44, 46–49). Moreover, using a serial analysis of gene expression approach, STC1 was found to be induced in human glioblastoma cells under hypoxia conditions (50). Together with the recent studies on STC1 in cancer biology, it was therefore anticipated that HIF-1 might have an effect on STC1 expression in cancer cells.

We have shown that HIF-1α directly stimulates STC1 gene expression. Using different hypoxic approaches in CNE-2 cells (*i.e.* the DFX, $CoCl_2$, and O_2 depletion), HIF-1α was activated, and STC1 expression was significantly increased. RNA interference showed that the induction of STC1 expression was HIF-1α dependent. Based on our Act D studies, we demonstrated that the induction of STC1 mRNA most likely occurred at the transcriptional level.

In DFX-treated CNE-2 cells with SNP and/or Fe replenishment, both HIF-1α and STC1 mRNA levels were substantially reduced. The three compounds [SNP, $K_3Fe(CN)_6$, and $FeCl_3$] used in this study to suppress DFX-induced HIF-1α activity all contain iron. The release of ionic iron antagonized the Fe-chelating effect of DFX. Consequently, these treatments likely restored HIF prolyl hydroxylases activity and reactivated the process of HIF-1α degradation (30). Unlike $K_3Fe(CN)_6$ and $FeCl_3$, SNP is also a NO donor. The release of NO is known to inhibit the activation step of converting HIF-1α to its DNA-binding form (40). Moreover, the data indicated that SNP was more potent than $K_3Fe(CN)_6$ (a compound structurally related to SNP but without an NO group) in suppressing HIF-1α activity and STC1 expression.

Prior studies have demonstrated that hypoxia and VEGF treatment both elevate STC1 mRNA levels in several animal and cell line models. Cerebral ischemic biopsies from human and rat brain showed high level of STC1 expression (51). Biopsy specimens of pimonidazole-marked oropharyngeal carcinomas involving tissue hypoxia also have elevated STC1 levels (50). A study of the hypoxia-driven angiogenic pathways in von Hippel-Lindau syndrome-associated pheochromocytomas revealed that STC1 is one of the up-regulated candidate genes (52). Furthermore, hypoxia-induced STC1 mRNA expression has been reported in mouse embryonic fibroblasts (53). Although hypoxia was shown to induce STC1 expression in these studies, they did not provide direct evidence to indicate that the induction was HIF-1 dependent. During manuscript preparation, Manalo *et al.* (54) reported STC1 as one of the genes up-regulated in vascular endothelial cells under either hypoxic conditions or normoxia after infection with adenovirus encoding an active form of HIF-1α. Similarly, these data inferred but did not prove the direct involvement of HIF-1α in the regulation of STC1 expression. Using RNA interference, we have revealed that endogenous HIF-1α plays an essential role in hypoxia-induced STC1 expression. Thus, the STC1 gene is a possible target of HIF-1. It is well known that HIF-1 can also induce VEGF expression. Differential gene expression studies in vascular endothelial cells has demonstrated that the STC1 is indeed activated by VEGF treatment (23, 26–28), although no

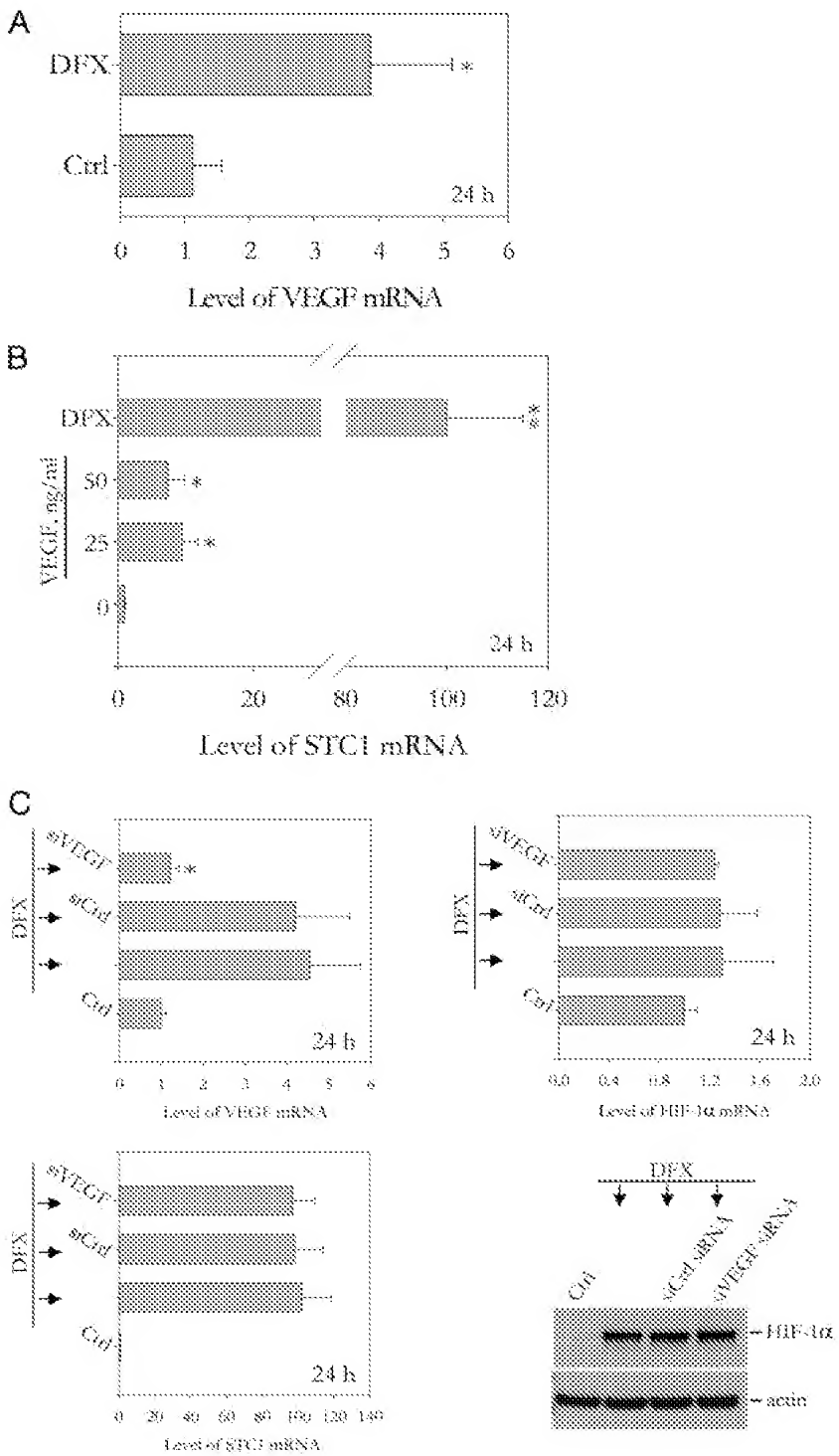


FIG. 5. Effect of VEGF on DFX-induced STC1 mRNA expression. A, Induction of VEGF mRNA at 24 h in DFX-treated CNE-2 cells. B, Induction of STC1 mRNA at 24 h in VEGF-treated (25–50 ng/ml) CNE-2 cells. *, $P < 0.01$, compared with the control. Note that the VEGF induction of STC1 mRNA is considerably lower than that of 50 μM DFX (#, $P < 0.01$). C, Effect of siRNA$_{VEGF}$ on VEGF, STC1, and HIF-1α mRNA levels at 24 h in DFX-treated CNE-2 cells. Western blot analysis revealed no significant reductions in HIF-1α protein levels in siRNA-treated cells. *, $P < 0.01$, compared with DFX treatment alone. Ctrl, Control.

experimental data have delineated the underlying mechanism. Consistent with these findings, our data also demonstrated that VEGF treatment alone stimulates STC1 gene expression. However, the magnitude of induction was markedly lower than that in hypoxic cells. Furthermore, the VEGF RNA interference and DFX/VEGF cotreatment studies revealed that VEGF had no significant effect on STC1 expression in DFX-treated cells, whereas HIF-1 had an overwhelming effect on STC1 gene activation. These findings support the notion that HIF-1 is a more potent stimulator of STC1 expression.

In summary, we have shown that hypoxia is an inducer of STC1 gene expression in various human cancer cell lines. More importantly, the present findings provide evidence that HIF-1 is a potent regulator of STC1 expression. The data obtained from SNP and Fe replenishment and RNA interference assays were unequivocal of the involvement of HIF-1 in the up-regulation of STC1 mRNA under hypoxic condi-

tions. Hypoxia-driven cellular responses can lead to apoptosis, growth arrest, and tumor vascularization (55–57). Fe chelation is able to up-regulate HIF-1α protein and Ndrg1, which is recognized as a metastatic suppressor gene that inhibits tumor growth (30, 36). A considerable number of studies and clinical trials have shown that DFX and other Fe chelators are effective antitumor agents (37, 39, 58–60). Fe chelation is demonstrated to be effective in causing cell arrest and apoptosis because tumor cells are far more sensitive than normal cells to Fe depletion (36, 37, 58, 61, 62). In hypoxic CNE-2 cells, the stimulation of STC1 expression was accompanied by a marked up-regulation of Ndrg1. The coactivation of STC1 and Ndrg1 expressions has also been demonstrated in one other study involving hypoxia in human carcinomas (50). Most solid human tumors have common hypoxic regions in the latter stages of carcinogenesis. Although the exact function of STC1 remains uncertain, it is possible that the HIF-1-mediated activation of STC1 relates to the final stages of cancer development.

Acknowledgments

Received March 29, 2005. Accepted August 10, 2005.

Address all correspondence and requests for reprints to: Dr. Chris K. C. Wong, Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong.

This work was supported by the Research Grants Council, Hong Kong, and the Faculty Research Grant, Hong Kong Baptist University (to C.K.C.W.) and through funding from The Canadian Institutes of Health Research and the Kidney Foundation of Canada (to G.F.W.).

References

1. **Wagner GF, Hampong M, Park CM, Copp DH** 1986 Purification, characterization, and bioassay of teleocalcin, a glycoprotein from salmon corpuscles of Stannius. Gen Comp Endocrinol 63:481–491
2. **Chang AC, Jeffrey KJ, Tokutake Y, Shimamoto A, Neumann AA, Dunham MA, Cha J, Sugawara M, Furuichi Y, Reddel RR** 1998 Human stanniocalcin (STC): genomic structure, chromosomal localization, and the presence of CAG trinucleotide repeats. Genomics 47:393–398
3. **Varghese R, Wong CK, Deol H, Wagner GF, DiMattia GE** 1998 Comparative analysis of mammalian stanniocalcin genes. Endocrinology 139:4714–4725
4. **Chang AC, Janosi J, Hulsbeek M, de Jong D, Jeffrey KJ, Noble JR, Reddel RR** 1995 A novel human cDNA highly homologous to the fish hormone stanniocalcin. Mol Cell Endocrinol 112:241–247
5. **Olsen HS, Cepeda MA, Zhang QQ, Rosen CA, Vozzolo BL** 1996 Human stanniocalcin: a possible hormonal regulator of mineral metabolism. Proc Natl Acad Sci USA 93:1792–1796
6. **Chang AC, Jellinek DA, Reddel RR** 2003 Mammalian stanniocalcins and cancer. Endocr Relat Cancer 10:359–373
7. **Ishibashi K, Imai M** 2002 Prospect of a stanniocalcin endocrine/paracrine system in mammals. Am J Physiol Renal Physiol 282:F367–F375
8. **Filvaroff EH, Guillet S, Zlot C, Bao M, Ingle G, Steinmetz H, Hoeffel J, Bunting S, Ross J, Carano RA, Powell-Braxton L, Wagner GF, Eckert R, Gerritsen ME, French DM** 2002 Stanniocalcin 1 alters muscle and bone structure and function in transgenic mice. Endocrinology 143:3681–3690
9. **Varghese R, Gagliardi AD, Bialek PE, Yee SP, Wagner GF, DiMattia GE** 2002 Overexpression of human stanniocalcin affects growth and reproduction in transgenic mice. Endocrinology 143:868–876
10. **McCudden CR, James KA, Hasilo C, Wagner GF** 2002 Characterization of mammalian stanniocalcin receptors. Mitochondrial targeting of ligand and receptor for regulation of cellular metabolism. J Biol Chem 277:45249–45258
11. **Fujiwara Y, Emi M, Ohata H, Kato Y, Nakajima T, Mori T, Nakamura Y** 1993 Evidence for the presence of two tumor suppressor genes on chromosome 8p for colorectal carcinoma. Cancer Res 53:1172–1174
12. **Fujiwara Y, Monden M, Mori T, Nakamura Y, Emi M** 1993 Frequent multiplication of the long arm of chromosome 8 in hepatocellular carcinoma. Cancer Res 53:857–860
13. **Trapman J, Sleddens HF, van der Weiden MM, Dinjens WN, Konig JJ, Schroder FH, Faber PW, Bosman FT** 1994 Loss of heterozygosity of chromosome 8 microsatellite loci implicates a candidate tumor suppressor gene between the loci D8S87 and D8S133 in human prostate cancer. Cancer Res 54:6061–6064
14. **Kagan J, Stein J, Babaian RJ, Joe YS, Pisters LL, Glassman AB, von Eschenbach AC, Troncoso P** 1995 Homozygous deletions at 8p22 and 8p21 in prostate cancer implicate these regions as the sites for candidate tumor suppressor genes. Oncogene 11:2121–2126
15. **Takle LA, Knowles MA** 1996 Deletion mapping implicates two tumor suppressor genes on chromosome 8p in the development of bladder cancer. Oncogene 12:1083–1087
16. **Fujiwara Y, Sugita Y, Nakamori S, Miyamoto A, Shiozaki K, Nagano H, Sakon M, Monden M** 2000 Assessment of Stanniocalcin-1 mRNA as a molecular marker for micrometastases of various human cancers. Int J Oncol 16:799–804
17. **Gerritsen ME, Soriano R, Yang S, Ingle G, Zlot C, Toy K, Winer J, Draksharapu A, Peale F, Wu TD, Williams PM** 2002 *In silico* data filtering to identify new angiogenesis targets from a large *in vitro* gene profiling data set. Physiol Genomics 10:13–20
18. **Okabe H, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y, Tsunoda T, Furukawa Y, Nakamura Y** 2001 Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. Cancer Res 61:2129–2137
19. **Watanabe T, Ichihara M, Hashimoto M, Shimono K, Shimoyama Y, Nagasaka T, Murakumo Y, Murakami H, Sugiura H, Iwata H, Ishiguro N, Takahashi M** 2002 Characterization of gene expression induced by RET with MEN2A or MEN2B mutation. Am J Pathol 161:249–256
20. **McCudden CR, Majewski A, Chakrabarti S, Wagner GF** 2004 Co-localization of stanniocalcin-1 ligand and receptor in human breast carcinomas. Mol Cell Endocrinol 213:167–172
21. **Welcsh PL, Lee MK, Gonzalez-Hernandez RM, Black DJ, Mahadevappa M, Swisher EM, Warrington JA, King MC** 2002 BRCA1 transcriptionally regulates genes involved in breast tumorigenesis. Proc Natl Acad Sci USA 99: 7560–7565
22. **Bouras T, Southey MC, Chang AC, Reddel RR, Willhite D, Glynne R, Henderson MA, Armes JE, Venter DJ** 2002 Stanniocalcin 2 is an estrogen-responsive gene coexpressed with the estrogen receptor in human breast cancer. Cancer Res 62:1289–1295
23. **Kahn J, Mehraban F, Ingle G, Xin X, Bryant JE, Vehar G, Schoenfeld J, Grimaldi CJ, Peale F, Draksharapu A, Lewin DA, Gerritsen ME** 2000 Gene expression profiling in an *in vitro* model of angiogenesis. Am J Pathol 156: 1887–1900
24. **Ismail RS, Baldwin RL, Fang J, Browning D, Karlan BY, Gasson JC, Chang DD** 2000 Differential gene expression between normal and tumor-derived ovarian epithelial cells. Cancer Res 60:6744–6749
25. **Liang P, Averboukh L, Keyomarsi K, Sager R, Pardee AB** 1992 Differential display and cloning of messenger RNAs from human breast cancer versus mammary epithelial cells. Cancer Res 52:6966–6968
26. **Bell SE, Mavila A, Salazar R, Bayless KJ, Kanagala S, Maxwell SA, Davis GE** 2001 Differential gene expression during capillary morphogenesis in 3D collagen matrices: regulated expression of genes involved in basement membrane matrix assembly, cell cycle progression, cellular differentiation and G-protein signaling. J Cell Sci 114:2755–2773
27. **Liu D, Jia H, Holmes DI, Stannard A, Zachary I** 2003 Vascular endothelial growth factor-regulated gene expression in endothelial cells: KDR-mediated induction of Egr3 and the related nuclear receptors Nur77, Nurr1, and Nor1. Arterioscler Thromb Vasc Biol 23:2002–2007
28. **Wary KK, Thakker GD, Humtsoe JO, Yang J** 2003 Analysis of VEGF-responsive genes involved in the activation of endothelial cells. Mol Cancer 2:25
29. **Wascher RA, Huynh KT, Giuliano AE, Hansen NM, Singer FR, Elashoff D, Hoon DS** 2003 Stanniocalcin-1: a novel molecular blood and bone marrow marker for human breast cancer. Clin Cancer Res 9:1427–1435
30. **Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ** 2001 Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 292:468–472
31. **Kraggerud SM, Sandvik JA, Pettersen EO** 1995 Regulation of protein synthesis in human cells exposed to extreme hypoxia. Anticancer Res 15:683–686
32. **Pettersen EO, Juul NO, Ronning OW** 1986 Regulation of protein metabolism of human cells during and after acute hypoxia. Cancer Res 46:4346–4351
33. **Huang LE, Gu J, Schau M, Bunn HF** 1998 Regulation of hypoxia-inducible factor 1alpha is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA 95:7987–7992
34. **Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ** 1999 The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399:271–275
35. **Garayoa M, Martinez A, Lee S, Pio R, An WG, Neckers L, Trepel J, Montuenga LM, Ryan H, Johnson R, Gassmann M, Cuttitta F** 2000 Hypoxia-inducible factor-1 (HIF-1) up-regulates adrenomedullin expression in human tumor cell lines during oxygen deprivation: a possible promotion mechanism of carcinogenesis. Mol Endocrinol 14:848–862
36. **Le NT, Richardson DR** 2004 Iron chelators with high antiproliferative activity up-regulate the expression of a growth inhibitory and metastasis suppressor gene: a link between iron metabolism and proliferation. Blood 104:2967–2975

37. **Chaston TB, Lovejoy DB, Watts RN, Richardson DR** 2003 Examination of the antiproliferative activity of iron chelators: multiple cellular targets and the different mechanism of action of triapine compared with desferrioxamine and the potent pyridoxal isonicotinoyl hydrazone analogue 311. Clin Cancer Res 9:402–414
38. **Cooper CE, Lynagh GR, Hoyes KP, Hider RC, Cammack R, Porter JB** 1996 The relationship of intracellular iron chelation to the inhibition and regeneration of human ribonucleotide reductase. J Biol Chem 271:20291–20299
39. **Liang SX, Richardson DR** 2003 The effect of potent iron chelators on the regulation of p53: examination of the expression, localization and DNA-binding activity of p53 and the transactivation of WAF1. Carcinogenesis 24:1601–1614
40. **Sogawa K, Numayama-Tsuruta K, Ema M, Abe M, Abe H, Fujii-Kuriyama Y** 1998 Inhibition of hypoxia-inducible factor 1 activity by nitric oxide donors in hypoxia. Proc Natl Acad Sci USA 95:7368–7373
41. **Cadenas E, Poderoso JJ, Antunes F, Boveris A** 2001 Analysis of the pathways of nitric oxide utilization in mitochondria. Free Radic Res 33:747–756
42. **Chen W, Jin W, Tian H, Sicurello P, Frank M, Orenstein JM, Wahl SM** 2001 Requirement for transforming growth factor β1 in controlling T cell apoptosis. J Exp Med 194:439–453
43. **Wrutniak-Cabello C, Casas F, Cabello G** 2001 Thyroid hormone action in mitochondria. J Mol Endocrinol 26:67–77
44. **Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, Ratcliffe PJ** 1997 Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci USA 94:8104–8109
45. **Wang GL, Jiang BH, Rue EA, Semenza GL** 1995 Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci USA 92:5510–5514
46. **Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL, Bedi A** 2000 Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. Genes Dev 14:34–44
47. **Akakura N, Kobayashi M, Horiuchi I, Suzuki A, Wang J, Chen J, Niizeki H, Kawamura K, Hosokawa M, Asaka M** 2001 Constitutive expression of hypoxia-inducible factor-1α renders pancreatic cancer cells resistant to apoptosis induced by hypoxia and nutrient deprivation. Cancer Res 61:6548–6554
48. **Jiang BH, Agani F, Passaniti A, Semenza GL** 1997 V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. Cancer Res 57:5328–5335
49. **Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E** 1998 Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 394:485–490
50. **Lal A, Peters H, St. Croix B, Haroon ZA, Dewhirst MW, Strausberg RL, Kaanders JH, van der Kogel AJ, Riggins GJ** 2001 Transcriptional response to hypoxia in human tumors. J Natl Cancer Inst 93:1337–1343
51. **Zhang K, Lindsberg PJ, Tatlisumak T, Kaste M, Olsen HS, Andersson LC** 2000 Stanniocalcin: a molecular guard of neurons during cerebral ischemia. Proc Natl Acad Sci USA 97:3637–3642
52. **Eisenhofer G, Huynh TT, Pacak K, Brouwers FM, Walther MM, Linehan WM, Munson PJ, Mannelli M, Goldstein DS, Elkahloun AG** 2004 Distinct gene expression profiles in norepinephrine- and epinephrine-producing hereditary and sporadic pheochromocytomas: activation of hypoxia-driven angiogenic pathways in von Hippel-Lindau syndrome. Endocr Relat Cancer 11:897–911
53. **Ito D, Walker JR, Thompson CS, Moroz I, Lin W, Veselits ML, Hakim AM, Fienberg AA, Thinakaran G** 2004 Characterization of stanniocalcin 2, a novel target of the mammalian unfolded protein response with cytoprotective properties. Mol Cell Biol 24:9456–9469
54. **Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, Garcia JG, Semenza GL** 2005 Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood 105:659–669
55. **Giatromanolaki A, Sivridis E, Koukourakis MI** 2004 Tumour angiogenesis: vascular growth and survival. APMIS 112:431–440
56. **Schmitt O, Schubert C, Feyerabend T, Hellwig-Burgel T, Weiss C, Kuhnel W** 2002 Preferential topography of proteins regulating vascularization and apoptosis in a MX1 xenotransplant after treatment with hypoxia, hyperthermia, ifosfamide, and irradiation. Am J Clin Oncol 25:325–336
57. **Hopfl G, Wenger RH, Ziegler U, Stallmach T, Gardelle O, Achermann R, Wergin M, Kaser-Hotz B, Saunders HM, Williams KJ, Stratford IJ, Gassmann M, Desbaillets I** 2002 Rescue of hypoxia-inducible factor-1α-deficient tumor growth by wild-type cells is independent of vascular endothelial growth factor. Cancer Res 62:2962–2970
58. **Le NT, Richardson DR** 2002 The role of iron in cell cycle progression and the proliferation of neoplastic cells. Biochim Biophys Acta 1603:31–46
59. **Richardson DR** 2001 The controversial role of deferiprone in the treatment of thalassemia. J Lab Clin Med 137:324–329
60. **Richardson DR, Ponka P** 1998 Pyridoxal isonicotinoyl hydrazone and its analogs: potential orally effective iron-chelating agents for the treatment of iron overload disease. J Lab Clin Med 131:306–315
61. **Darnell G, Richardson DR** 1999 The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents III: the effect of the ligands on molecular targets involved in proliferation. Blood 94:781–792
62. **Brodie C, Siriwardana G, Lucas J, Schleicher R, Terada N, Szepesi A, Gelfand E, Seligman P** 1993 Neuroblastoma sensitivity to growth inhibition by desferrioxamine: evidence for a block in G_1 phase of the cell cycle. Cancer Res 53:3968–3975

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

American Journal of Pathology, Vol. 156, No. 6, June 2000

Gene Expression Profiling in an *in Vitro* Model of Angiogenesis

Jeanne Kahn,* Fuad Mehraban,† Gladys Ingle,* Xiaohua Xin,* Juliet E. Bryant,† Gordon Vehar,‡ Jill Schoenfeld,* Chrisopher J. Grimaldi,§ Franklin Peale,¶ Aparna Draksharapu,¶ David A. Lewin,† and Mary E. Gerritsen*

From the Departments of Cardiovascular Research, Protein Chemistry,‡ Molecular Biology,§ and Pathology,¶ Genentech, South San Francisco, California; and Curagen Corporation,† New Haven, Connecticut*

In the present study we have used a novel, comprehensive mRNA profiling technique (GeneCalling) for determining differential gene expression profiles of human endothelial cells undergoing differentiation into tubelike structures. One hundred fifteen cDNA fragments were identified and shown to represent 90 distinct genes. Although some of the genes identified have previously been implicated in angiogenesis, potential roles for many new genes, including OX-40, white protein homolog, KIAA0188, a homolog of angiopoietin-2, ADAMTS-4 (aggrecanase-1), and stanniocalcin were revealed. Support for the biological significance was confirmed by the abrogation of the changes in the expression of angiogenesis inhibitors and *in situ* hybridization studies. This study has significantly extends the molecular fingerprint of the changes in gene expression that occur during endothelial differentiation and provides new insights into the potential role of a number of new molecules in angiogenesis. *(Am J Pathol 2000, 156:1887–1900)*

Angiogenesis, defined as the process whereby new blood vessels are formed from previously existing ones, plays an important role in the development and progression of a number of disease states, including various cancers, diabetic retinopathy, macular degeneration, psoriasis, and rheumatoid arthritis. During the last 10 years there have been many advances in our understanding of the biology of and the molecules that are involved in angiogenesis. A number of different growth factors, including vascular endothelial growth factor (VEGF), fibroblast growth factors, platelet-derived growth factor, hepatocyte growth factor, angiopoietins 1 and 2, as well as various endothelial surface molecules, such as CD31 (PECAM), CD144 (VE-Cadherin), and $\alpha v\beta 3$ integrins, have been implicated in various steps of angiogenesis. These advances have enabled the development of new therapeutic strategies for inhibiting angiogenesis (eg, to inhibit tumor growth) or promoting angiogenesis (coronary and peripheral ischemia, wound healing).

Understanding the molecular events that direct angiogenesis and the order in which they occur and identifying new pathways that are required for this process are of fundamental importance for all researchers who study angiogenesis. The present study was undertaken to identify the alterations in gene expression that occur in an *in vitro* model of angiogenesis. In this model, endothelial cells are suspended in a three-dimensional gel composed of type I collagen and incubated with a mixture of stimuli (phorbol myristate acetate (PMA), basic fibroblast growth factor (bFGF), and vascular endothelial cell growth factor (VEGF)). Previous studies by our laboratory demonstrated that this combination of stimuli resulted in the optimal formation of a three-dimensional tubular network of endothelial cells with interconnecting lumenal structures.[1] In this model, endothelial differentiation into tubelike structures is completely blocked by inhibitors of new mRNA (actinomycin D) or protein synthesis (cycloheximide). Furthermore, the cells progress through this differentiation process in a coordinated and synchronized manner, thus optimizing the profile of gene expression.

The goal of the present study was to identify a molecular fingerprint or transcriptional profile of endothelial differentiation into tubelike structures, using amplification and an imaging approach called GeneCalling.[2] This method was previously shown to provide a comprehensive sampling of cDNA populations in conjunction with the sensitive detection of quantitative differences in mRNA abundance for both known and novel genes.[2] We describe the identification of 115 differentially expressed cDNA fragments, which corresponded to 90 previously identified genes. The identification and differential expression of these genes was confirmed by a second independent method employing real-time quantitative polymerase chain reaction (PCR). Although some of the cDNA fragments identified were genes previously known to play some role in the process of angiogenesis, many other differentially expressed genes were unexpected

Accepted for publication February 9, 2000.

Ms. Kahn and Dr. Mehraban contributed equally to this study.

Address reprint requests to Dr. Mary E. Gerritsen, Department of Cardiovascular Research, MS 42 Genenetch, 1 DNA Way, South San Francisco, CA 94080. E-mail: meg@gene.com.

and suggest possible roles for these additional genes in endothelial differentiation and vessel assembly.

Materials and Methods

Materials

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA) and maintained in endothelial growth medium (EGM) media supplemented to a final concentration of 10% fetal bovine serum. Type I rat tail collagen was from Upstate Biotechnology (Lake Success, NY), and recombinant bFGF was purchased from Collaborative Biomedical Products (Becton Dickinson Labware, Bedford, MA). Recombinant VEGF was from Genentech (South San Francisco, CA). Medium 199 (10×) (M199, M0650), PMA, ITS (insulin, transferrin, and selenium-A), trypsin, actinomycin D, and cycloheximide were from Gibco-BRL (Gaithersburg, MD).

Formation of Three-Dimensional Collagen Gels

Collagen gels were formed by mixing together ice-cold gelation solution (10× M199, H_2O, 0.53 mol/L $NaHCO_3$, 200 mmol/L L-glutamine, type I collagen, 0.1 mol/L NaOH, 100:27.7:50:10:750:62.5 v/v) and cells in 1× basal medium (see below) at a concentration of 3×10^6 cells/ml at a ratio of four volumes gelation solution to one volume of cells. The gels were allowed to form by incubation in a CO_2-free incubator at 37°C for 30 minutes to 1 hour. The gels were then overlaid with 1× basal medium consisting of M199 supplemented with 1% FBS, 1× ITS, 2 mmol/L L-glutamine, 50 μg/ml ascorbic acid, 26.5 mmol/L $NaHCO_3$, 100 U/ml penicillin, and 100 U/ml streptomycin. In the tube-forming experiments, the culture medium was supplemented with 80 nmol/L PMA, 40-ng/ml bFGF, and 40 ng/ml VEGF.

mRNA Isolation and cDNA Synthesis

Medium was aspirated from the surface of the collagen gels, and the gels were scraped into a 50-ml polypropylene tube containing three volumes of Tri-Reagent-LS (Molecular Research Center, Cincinnati, OH). The tubes were incubated for 10 minutes at 23°C with intermittent gentle agitation. The tubes were stored at −80°C until all experimental samples had been collected. The tubes were then thawed at room temperature, and the RNA was extracted following the manufacturer's specifications. The RNA pellets were resuspended in diethyl-pyrolidine-carbonate-treated water, and the RNA content was quantified spectroscopically at 260 nm. RNA samples were stored at −20°C. Samples used for GeneCalling analysis were shipped on dry ice to CuraGen (New Haven, CT). Samples from time points of 4, 24, and 48 hours were used for the GeneCalling analysis, and in separate experiments, samples from additional time points of 30 minutes and 2, 4, 8, 16, 24, 38, and 46.5 hours were prepared for TaqMan confirmation. For the quantitative expression analysis, contaminating DNA was removed by treatment of the isolated RNA with DNase I (Promega, Madison, WI). PolyA+ RNA was prepared by fractionation of total RNA with an mRNA purification kit that uses the biotinylated oligo-dT-streptavidin magnetic bead method (MPG, Lincoln Park, NJ), followed by cDNA synthesis by reverse transcription of oligo-dT-primed mRNA (Superscript II; Life Technologies) and second-strand synthesis. Terminal phosphate removal is achieved by treatment with arctic shrimp alkaline phosphatase (Amersham Life Sciences, Piscataway, NJ), followed by purification of cDNA by phenol-chloroform extraction. Yield of cDNA was quantitated by fluorometry using PicoGreen dye (Molecular Probes, Eugene, OR). Double-stranded DNA was digested using pairs of restriction enzymes with 6-bp recognition sites. More than 48 enzyme pairs were used and were chosen such that a representative coverage of most of the possible sequences in a given DNA sample was achieved.[2] PCR amplification using specific linkers was carried out as described previously.[2] The final DNA products were denatured by heating to 96°C and electrophoresed on ultrathin polyacrylamide gels under denaturing conditions in 6 mol/L urea. PCR products were visualized by the presence of 6-carboxy fluorescein (FAM) label on the product, using a multicolor laser excitation (Niagara; CuraGen, New Haven CT) imaging system.

Data Interpretation

The data obtained from Niagara gels were queried (ie, "GeneCalled") against public and proprietary databases.[2] GeneCalling is the process that takes the restriction enzyme pair recognition site information and the cDNA fragment size determined from the migration of the labeled fragment on Niagara gels and uses that information (the size of the fragment and the relative position of the terminal sequences defined by the restriction enzyme pairs) to search public and proprietary databases for likely gene matches, using statistical and mathematical criteria. A GeneCall is defined as the probability of a cDNA fragment belonging to a known gene.[2] The cDNA fragment data were compiled as a list of likely genes to which that cDNA fragment might belong. If a provisional identification of a cDNA fragment could not be obtained by querying databases, the cDNA fragment was designated as belonging to a putative novel gene.

Confirmation of Gene Calls

GeneCalls were confirmed in a competitive PCR reaction, "GeneCall poisoning," in which the known sequence of the likely gene of interest is used to design poisoning primers as previously described.[2] Ablation of the cDNA fragment of interest confirmed that the cDNA fragment belonged to the gene for which the specific poisoning primer was designed.

Novel cDNA Fragments

If no GeneCall was obtained for a cDNA fragment, the cDNA fragment was eluted and subcloned into *Esche-*

richia coli with the standard TA-cloning vector (Invitrogen, Palo Alto, CA). The cDNA fragment was then sequenced, and the resulting sequence was used to design poisoning primers for confirmation as described above.

Validation and Confirmation of Gene Expression by Quantitative Reverse Transcriptase-Polymerase Chain Reaction (TaqMan)

To confirm the expression data from GeneCalling by an independent technique, gene-specific PCR oligonucleotide primer pairs and an oligonucleotide probe labeled with a reporter fluorescent dye at the 5′ end and quencher fluorescent dye at the 3′ end were designed using Oligo 4.0 software (National Bioscience, Plymouth, MN). Table 1 provides the sequences for the primers and probes used in this study. Total RNA (50 ng) was added to a 50 μl reverse transcriptase-polymerase chain reaction (RT-PCR) reaction mixture according to the manufacturer's protocol (Roche Molecular Systems, Branchburg, NJ). The thermal cycling conditions included one cycle at 48°C for 30 minutes, one cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 s, annealing at 60°C for 1 minute, and a final hold at 25°C for 2 minutes. Standard curves for the expression of each gene were generated by serial dilution of a standard preparation of total RNA isolated from quiescent HUVECs grown in monolayer culture. Data are expressed as the fold induction normalized to the same gene from quiescent HUVEC RNA.

Treatment with 15d-PGJ$_2$

mRNA was harvested from endothelial cells incubated 4 and 24 hours in the absence (control) or presence of 10 μmol/L of the PPARγ ligand 15-deoxy-Δ2,14–15-prostaglandin J_2 (15d-PGJ_2). In both groups the cells were incubated with the mixture of growth stimuli (ie, PMA, VEGF, and bFGF), and the cells were incorporated in the collagen gels as described above.

In Situ *Hybridization of Tissue Specimens*

Formalin-fixed, paraffin-embedded human tissues were investigated for *in situ* mRNA expression. Tissues included first-trimester (14–15-week) placenta, adult adrenal cortex, aorta, muscular artery with atherosclerosis, brain, gall bladder, heart, pancreas, prostate, stomach, eye with age-related macular degeneration (AMD), inflamed appendix, pulmonary adenocarcinoma, ductal mammary adenocarcinoma, kidney with renal cell carcinoma, hepatocellular carcinoma, squamous cell carcinoma, osteosarcoma, and chondrosarcoma. *In vitro* transcription and ^{33}P labeling of sense and antisense riboprobes were performed as described previously.[3] Briefly, stanniocalcin, osteonidogen, podocalyxin, and ADAMTS-4 sequences were PCR-amplified from plasmid DNA, using gene-specific primers that encoded T3 or T7 RNA polymerase initiation sites. Sense and antisense riboprobes were prepared by *in vitro* transcription from the PCR-amplified templates and diluted in hybridization buffer to a specific activity of 1×10^6 cpm/ml. Tissue sections 5 μm thick were deparaffinized, deproteinated in 4 μg/ml of proteinase K for 30 minutes at 37°C, hybridized at 55°C overnight, then washed at high stringency (55°C in 0.1× standard saline citrate for 2 hours). Glass slides were dipped in NBT2 nuclear track emulsion (Eastman Kodak), exposed in sealed plastic slide boxes containing desiccant for 4 weeks at 4°C, developed, and counterstained with hematoxylin and eosin.

Results

cDNA Fragment Selection and Identification

As reported previously,[1] incubation of endothelial cells in 3D gels in the absence of the growth factors resulted in rapid induction of apoptosis. Therefore, no comparison was made of mRNA from cells in 3D gels in the absence of growth factors. Instead we evaluated temporal changes in gene expression in the 3D gel environment in the presence of PMA, VEGF, and bFGF, by comparison of the RNA harvested at 4, 24, and 48 hours. A summary of the differences observed can be found in Table 2. The differentially expressed cDNA fragments in the 24-hour *versus* the 4-hour data set were examined in more detail. As shown in Table 3, the identities of 115 cDNA fragments were determined by oligonucleotide poisoning or cloning of the gene fragments, resulting in the identification of 90 distinct genes. In addition (not shown), 80 cDNA fragments were identified as totally novel or as corresponding to expressed sequence tags (ESTs) of unknown function. Full-length cloning of these genes is currently under way.

Confirmation of cDNA Fragment Identification and Expression

TaqMan probes were prepared to confirm by an independent method the identification of 67 of the genes identified by GeneCalling. As shown in Table 3, there was a reasonable agreement in the direction of the fold induction as predicted by GeneCalling and as analyzed by TaqMan. Disagreement between the GeneCalling and TaqMan results are likely due to small differences in the temporal sequence of events in the two separate experiments (ie, the RNA harvested for the GeneCalling experiment *versus* the RNA harvested for later TaqMan analysis), which could readily account for the discrepancies in the fold induction. A more complete time course analysis of 26 of the genes is depicted in Figure 1, A–D.

Genomic Response of Endothelial Cells in a Tube-Forming Environment

According to the classification schema used, the genes identified fell into most of the major role categories, including cell division, cell signaling, cell adhesion, hormone/growth factors, receptors, cytoskeleton, extracellu-

Table 1. Taqman Primer and Probes Sets

Gene name	Forward primer	Reverse primer	Probe
Hormones/growth factors			
Placental growth factor (PLGF)	GACGTTCTCTCAGCACGTTCG	CACCTTTCCGGCTTCATCTTC	CGAATGCCGGCCTCTGCGG
Stanniocalcin precursor	CGAGTGGCGGCTCAAAA	CCGCAGCCGACCTGTAGA	TCAGCTGAAGTGGTTCGTTGCCTCAA
Fibroblast growth factor 16 (FGF-16)	CCTTAGCTGACTCCCCAGGTT	CTGCAGCTTCCCCTCGATT	CCTGAACGAGCGCCTGGGCC
Angiopoeitin-2 Hlog (ai079861)	GGCCTGCAGCGGGTAGTA	GGCAGAAGCTTAAGAAGGGAATC	CGGCCCCGCCAGGTCTTCC
Connective tissue growth factor	TGCACCGCCAAAGATGGT	GGACTCTCCGCTGCGGTAC	CTCCCTGCATCTTCGGTGGTACGG
Cell cycle/apoptosis			
Human replication factor C	TTGCTTGTAATGCTTCGGATAAGA	TGTGTACCGGAGGACTGCAC	CATCGAGCCCATTCAGTCCCGC
ALG-2 interacting protein	TGAGCAATGGATCTGTTAACCAA	TCCCGTGTCAGTACAGCAGTCT	TCGGCTTTCCTCTGATTATAGGCAGCCA
p120	CCTCCGGACCAATACCTTGA	CCCCACGATTGATTAGAGCCT	CCCGACGCCGAGACCTTGCA
CDEBP (amyloid precursor-like protein 2)	GATGCCTCGTTGGTACTTCGA	CGCCGCAGCCACCATA	AAGCGCACGCACTTTCCCTTGG
BCL-2 related A1 protein	CAGCTCAAGACTTTGCTCTCCA	AGTCCTGAGCCAGCCTGTAAAT	ATCCAAATTCACAGTCTGTCATCTTCTGCCTG
CDC28/CDC2 associate protein CLK	TTGTTGCCTGGGCTGGA	TGAAACGAGAGCGCGAAGA	CGGGCGCAGCAGACAAAACCA
Human binding protein	GGCTACATCGAGGCTCTTGC	GGCTACATCGAGGCTCTTGC	GGCTACATCGAGGCTCTTGC
Polo-like kinase	GGATCACACCAAGCTCATCTTG	CCCGCTTCTCGTCGATGT	CCCACTGATGGCAGCCGTGACC
p53	GAGGTGCGTGTTTGTGCCT	TTCTTGCGGAGATTCTCTTCCT	TGCGCCGGTCTCTCCCAGGA
DNA binding proteins/transcription factors/histones/repair			
OS-9	AAGGCCTCCAAGCAGCATC	GGTCGCACTTGGACCCATT	TCTTAAACGCTACCACAGCCAGACCTATGG
Mel-18 homolog	GCGCTTCCTGCGATGC	TGCGGAGAAACTTGGCAAG	CAGCAGCCATGACCGTCATGCA
RNA synthesis/ribosomes			
Clone 23689	CTCAGCCCTCCGAGACCA	CTCCCGGATAATCTTGAGCACT	CGTGGGCAACATGGTGCGGA
Ribosomal protein L37	TGTCCTGGCTGGACGCTACT	TGAGGTGCCATCATCAATGTTC	TCACGATGACAGCTTTGCGTCCG
Chemokines			
Interleukin-8 (IL-8)	AAGGAACCATCTCACTGTGTGTAAAC	ATCAGGAAGGCTGCCAAGAG	TGACTTCCAAGCTGGCCGTGGC
GRO-1α (MGSA)	TGAGGAGCCTGCAACATGC	CATTGGCCATTTGCTTGGA	CCGCCAGCCTCTATCACAGTGGCT
Tyrosine kinase receptors			
axl	GCATGAAGGAATTTGACCATCC	TCTCTCGTTCAGAACCCTGGA	CAGACACCGATGAGCCTCATGACGTT
Epithelial cell tyrosine kinase (ECK)	GCCTGTTCACCAAGATTGACAC	GCCTCGAAGTCGCTGCTG	TTGCGCCCGATGAGATCACCG
Serine/threonine kinases			
Serum-inducible kinase	GAGGATCGTCCCAGTTTGGA	GAAGACAGTCTGTCCGGAGTGAA	CATCATTCGACATGACTTTTTTTTGCAGGG
Branched chain alpha keto acid	CGGTTCCCCTTCATCCCTAT	TGTGGCTCTCATGGCATTCTT	CCACTGGACTACATCCTGCCGGAGCT
Dehydrogenase kinase			
Thymidylate kinase	AAGCASTCGAAGCTGTCCATGA	TCTCTGTGGCAGTGCGGAT	TCCGCGTGCTCTCTGAGGACGC
Other receptors/integral membrane glycoproteins			
OX40	CCAACTCTGCACCGTTCTAGG	GGTATGCATGGCATACGTAAGC	CCGATGGCTGCCTCCGGCT
CXCR-4	CGCTACCTGGCCATCGTC	CATAGACCACCTTTTCAGCCAAC	CGCCACCAACAGTCAGAGGCCA
Podocalyxin-like protein	GGGCATGGTGAGGTTTCATCT	TTTACGCCCAGAACGATGG	CCATGGCGAAAGTTCAACATTCCACA
Alpha-2 integrin	TCTGAGACTGCCAAGGTCTTCA	CAGCTGGTATTTGTCGGACATC	AGGACTAGATCAGAAATGCAAAGTCCATCCTCA
JuSo MUC18 glycoprotein	GAACACAGTGGGCGCTATGA	CCTGTGGTTCACTCAGCAGC	CAGGCCTGGAACTTGGACACCATGATAT
MHC class 1 antigen	GCGCTCCGCTACTACAACCA	CGTCGCAGCCAAACATCA	AGGCCGGTTCTCACACCCTCCAG
gp130	ATCCGCGCAAGATGTTGAC	ACCTGTAGATTCAGTGGTGAGGAAA	ACAAGGCTTGCACTACCCAAGTCTGCA
T-cell receptor Beta 2	GAGGGTCTCGGCCACCTT	AGAACTGGACTTGACAGCGGAA	TGGCAGAACCCCCGCAACCA
Protein zero-related protein	TGTGTCATATCAATTTCTGGATTCATAA	TTGATCCAACTGTGTCCAGAATG	TGACTTCGGCATTTATCCTTTGCTAATCTTGCT
Proteases/protease inhibitors			
Tissue factor pathway inhibitor-2 (TFPI-2)	CGATGCTTGCTGGAGGATAGA	ACACTGGTCGTCCACACTCACT	AAAGTTCCCAAAGTTTGCCGGCTGC
Aggrecanase (ADAMTS4; KIAA0688)	ACTGGTGGTGGCAGATGACA	TCACTGTTAGCAGGTAGCGCTTT	ATGGCCGCATTCCACGGTGC
Matrix metalloproteinase-9 (MMP-9)	CCCGGAGTGAGTTGAACCA	CCTAGTCCTCAGGGCACTGC	TGGACCAAGTGGGCTACGTGACCTATG
Matrix metalloproteinase-1 (MMP-1)	CATGAAAGGTGGACCAACAATTT	CCAAGAGAATGGCCGAGTTC	CAGAGTACAACTTACATCGTGTTGCGGCTCA

Table 1. Continued

Gene name	Forward primer	Reverse primer	Probe
Cathepsin B	GAAGCCATCTCTGACCGGATC	TCCGCCGACACCTCCA	CCACACCAATGCGCACGTCAGC
Plasminogen activator inhibitor-2 (PAI-2)	GCAGGCACAAGCTGCAGATA	CCTGTGGATGCATTGATTGC	TCCATTCATCCTTCCGCTCTCTCAGC
KIAA0188			
Transporter/channels			
yLAT1 glycoprotein amino acid	AGGAGGCAATGCCAGGAAG	CTTCATACTCAGTGCTGTCAACCA	TGGTGAAGGGTTTCCTCTCCTCCACC
Transporter			
White protein homolog	CCCTTTCAGATCATGTTCCCA	GGACGGCTGCGACGTC	CCAGTACACGATGCTGCAGTAGGCCA
Cytoskeleton/motility			
Moesin	ACTGGGCCGAGACAAATACAA	AATGCGCTGCTTGGTGTTG	CCCTGCGCCAGATCCGGC
actin bundling protein	CCAGCTGCTACTTTGACATCGA	CCATTGGACGCCCTCAGT	GATGCGCCGGTCACGCCA
T-plastin	AATAAAACAGCCATGCTCCCA	CCTTAAGCCATAAGCACTTCACC	TGCATGATTCGCAGGTCAGCTATTTCC
Brain ankyrin-2	AAGCAGCTTCCTGATGCATTC	CGGACACAGCGCCTTACAT	TCGCAGCCAAGAACAGCCACCA
Intermediate filaments			
Mesothelial keratin K7	CCCAGATCTCCGACACATCTG	GCGATGATGCCGTCCAG	CCATGGACAACAGTCGCTCCCTGG
Extracellular matrix			
Laminin gamma 2 (nicein B2 chain)	GCTGACAGGCAGGTGTTTGAA	CGAAGTAGCCTGCTTTGCACT	TGTATCCACAACACAGCCGGCATCTACTG
Nidogen-2 (osteonidogen)	AAAATCTTAGAACTTTTGTTGGGAAACTA	CCTTGACAGTTGGAGAAGCCA	AAATAATTGGTCCTTTCCCATCAGTTCTGCA
Type IV collagen	CCCCTGGAACCTCCTCTGTT	CATTGTGGTGCATCCGTTGT	CACATGGATTTCTTATTACACGCCACAGCC
Extracellular protein S1-5	GGAACCCAGCTGACCCTCA	CTGCTGCACACTGGATACGG	CGCATTCCCTCCAACCCTTCCC
p137	CAGTGCTCCCCGGGATTACT	CCAGAGCCTCGCTTGAAATT	TGCTGATATCCATCCCGTTGATAGCCA
Metabolic enzymes			
OXA1	ACACGGCTCCTATTCCCAGTAG	AAGCCGCAAGGAAGAGGTAGT	CCCGTGCTGCTGTCGCCCA
thioredoxin peroxidase AOE 37-2-187F	GAGGCATCCCGGGTATCG	GGCTTGGAAATCTTCGCTTTG	CGCCGACCACTCCCTGCACCTAA
Posttranslational protein modification			
Peptidyl-glycine alpha amidating monooxygenase	GAGGGTCTCGGCCACCTT	AGAACTGGACTTGACAGCGGAA	TGGCAGAACCCCCGCAACCA
Signal transduction			
TRAF-1	GGACCCATCTGATGCACCTT	TGTGGTCCTCGGATTGCTTT	TCCCTCACTCGATTCCCCGGG
Lipids and lipid turnover			
Phospholipase A_2 gamma	GAAGGCGGTGAGCCTGAAC	TTCCAGGGAGGTCCTGGTC	TTCTCGAGCATCTCAGTCAGCCAGGTG
Cyclooxygenase-2	GAATCATTCACCAGGCAAATTG	TCTGTACTGCGGGTGGAACA	TCCTACCACCAGCAACCCTGCCA
Coagulation system			
Tissue factor	CACCGACGAGATTGTGAAGGA	CCCTGCCGGGTAGGAGAA	ACCCGTGCCAAGTACGTCTGCTTCA
Clathrin components			
Clathrin heavy chain	GGAGAAAATTGTCCTTGATAACTCTGT	TCAGCCTTAATTGCAGTGAGGAT	TTCAGTGAACACAGGAATCTGCAAAACCTCC
Unknown			
ALG2 hlog	TGAGCAATGGATCTGTTAACCAA	TCCCGTGTCAGTACAGCAGTCT	TCGGCTTTCCTCTGATTATAGGCAGCCA
Sushi-like Repeat Protein	GGGCTTTCGATTGATTGGAAG	GGCAGTTCCAGACCAACGAC	TCGGTGCAATGCCTGCCAAGC

lar matrix, protein turnover, protein modification, and metabolism (Table 3). There was no apparent bias in the identification of any given class of gene.

The mRNA changes were clustered based on four basic patterns of expression. Group I, Early Transient (Figure 1A), which included interleukin-8 (IL-8), binding protein A1, plasminogen activator inhibitor-2 (PAI-2), growth-related oncogene α (GRO-α), and cyclooxygenase-2 (COX-2), was characterized by mRNA levels that were rapidly and highly induced then declined to the initial levels within 24 hours. Peak mRNA levels were observed at 2–4 hours. Not shown in Figure 1A because of the small magnitude of fold induction, is an EST with homology to the fibrinogen domain of angiopoeitin-2 (AI79861). The message levels for this EST increased by twofold by 4 hours then returned to baseline levels between 8 and 12 hours. Group II, Delayed Transient, which included the genes white protein homolog, fibroblast growth factor-16 (FGF16), KIAA0188, ADAMTS-4 (aggrecanase-1), tissue factor pathway inhibitor-2 (TFPI-2), podocalyxin-like protein, cathepsin B, and epithelial tyrosine kinase (ECK), was characterized by mRNA levels

Table 2. Summary of Statistics of the Gene Fragments Found to Be Differentially Modulated in GeneCalling at a Given Fold Difference Threshold

Comparison	Differentially expressed fragments (%)*	
	Difference threshold	
	±2-fold	±4-fold†
24 hrs *vs.* 4 hrs	1343 (4.8)	393 (1.4)
48 hrs *vs.* 24 hrs	1291 (4.7)	367 (1.4)
48 hrs *vs.* 4 hrs	368 (1.3)	27 (0.1)

*Value is the percentage of gene fragments observed to be differentially expressed in the comparison.
†Arbitrary higher cut-off.

that peaked somewhat later than those of Group I (8–12 hours), then fell back to near-baseline levels by 46.5 hours. Group III, Stable Induction (Figure 1C), was characterized by genes whose mRNA levels rise somewhat later than those of Group I or Group II, peaked at 12–46.5 hours, and remained markedly above baseline levels, even at 46.5 hours. This group included placental growth factor, yLAT1, clone 23689, osteonidogen, matrix metalloproteinase 9 (MMP-9), CXC chemokine receptor 4 (CXCR4), and stanniocalcin precursor (STC). Group IV, Rapid Repression (Figure 1D), was quite different from Groups I–III. mRNA levels for genes in Group IV declined from the initial value observed at 30 minutes and, by 16–24 hours, were below the mRNA levels observed in the mRNA controls obtained from quiescent HUVEcs (see Materials and Methods). Genes in Group IV included extracellular protein S1–5 (S1–5), axl, polo-like kinase, and mesothelial keratin.

PPARγ Modulation of Endothelial Gene Expression

Treatment of endothelial cells with 10 μmol/L 15-d-PGJ_2 completely blocks endothelial tube formation in response to bFGF, VEGF, and PMA.[4] As shown in Table 4, 15d-PGJ_2 treatment reduced or, in some cases, abrogated the fold increase (24 *versus* 4 hours) of PLGF, clone 23689, STC, OX-40, TFPI-2, MMP-9, KIAA0188, +yLAT1, laminin γ2, and $PLA_2\gamma$. In addition, 15d-PGJ_2 reduced the fold decrease (24 *versus* 4 hours) of IL-8, axl, PAI-2, white protein homolog, and keratin K-7 observed during tube formation. The ratios of other mRNAs (A1, FGF16, eck, podocalyxin, and osteonidogen, for example) were not markedly affected by 15d-PGJ_2.

In Situ *Hybridization Analyses*

As an additional test of the biological relevance of the genes identified, we evaluated the *in situ* expression of four of the genes identified in this study, ie, STC, podocalyxin, osteonidogen, and ADAMTS-4, by examining their expression in a number of different tumors as well as sections prepared from a variety of human organs (see Materials and Methods). All four genes were detected at sites of endothelial activation or new blood vessel formation. For example, STC demonstrated strong but variable expression in the vasculature in and around mammary adenocarcinoma and squamous cell carcinoma (Figure 2) and, to a lesser extent, in chondrosarcoma and renal cell carcinoma (not shown), but there was no significant expression seen in normal vessels (not shown). Detectable expression of podocalyxin in normal adult tissue was limited to glomerular urinary epithelial cells (podocytes) and some endothelial cells in the adventitia around large vessels. Podocalyxin expression was expressed in the endothelium of small vessels associated with chondrosarcoma, squamous and renal cell carcinomas, and ductal mammary adenocarcinoma. (not shown), as well as in arteriolar endothelium in inflamed appendix (Figure 2). Osteonidogen expression was absent in normal adult vessels but was observed in endothelial cells of inflamed appendix (not shown) and in peritumor stromal (Figure 2) endothelium and nonendothelial cell types, as well as in osteosarcoma, chondrosarcoma, and squamous cell CA tumors. ADAMTS-4 expression in adult tissue was intensely expressed in vascular endothelium and smooth muscle in areas of inflammation (appendices, around tumors, in inflamed lung) (Figure 2), as well as in scleral and corneal limbic endothelium in an age-related macular degeneration eye (not shown). No detectable expression of ADAMTS-4 was observed in blood vessels of normal adult tissues.

Discussion

Many of the genes identified in this experiment have previously been implicated in angiogenesis. For example, the mRNA for PLGF, a member of the VEGF family of growth factors, increased during the initial 8 hours of incubation in the gel environment (Figure 1C). The EST AI79861 identified from the GeneCalling analysis is 78% identical to the COOH-terminal region of human angiopoietin-2, a naturally occurring antagonist for the tie-2 receptor kinase,[5] suggesting that yet another member of the angiopoietin family may play some role in the regulation of new vessel formation.

TFPI-2, which was a highly up-regulated gene in this study (Figure 1B), is a 32-kd serine protease and is associated with the extracellular matrix that inhibits the activation of matrix metalloproteinase zymogens, pro-MMP-1 and MMP-3.[6,7] TFPI has also been reported to be a smooth muscle mitogen.[8] Thus the up-regulation of TFPI-2 by the differentiation of endothelial cells might have a dual role of limiting the extent of matrix degradation and recruiting or promoting the proliferation of mural cells, leading to the assembly of the new vessel wall.

The mRNA levels for a number of proteases increased substantially during the initial 8–10 hours of incubation in the gel, notably the matrix metalloproteinase, MMP-9 (Figure 1C), and cathepsin B (Figure 1B). MMP-9 is an established participant in angiogenesis, playing important roles in the degradation of basement membrane/matrix in both *in vitro* and *in vivo* models of angiogenesis.[9,10] MMP inhibitors reduce the elongation of endothelial cells into tubelike structures *in vitro*[11] and

Table 3. Genes Identified by GeneCalling

Confirmed gene*	Accesion no.†	GeneCalling ratio‡	TaqMan ratio§
Hormones/growth factors			
Placental growth factor	X54936	6	5
Transforming growth factor beta	X02812	11	2
Stanniocalcin precursor	U25997	14	8
FGF-16	AB009391	4	1
Angiopoetin-2 Hlog	AI079861	0.1	0.1
Connective tissue growth factor	U14750	0.1	0.2
Cell cycle/apoptosis			
Human replication factor C	M87338	5	23
ALG-2 interacting protein	sim to AJ005073	5	2
p120 proliferation associated antigen	X55504	4	1
CDEBP (amyloid precursor-like protein 2)	Z22572	4	1
Bcl-2 related protein A1	L19597	4	1
ABC50 ATP binding cassette protein	AF027302	2	ND
cdc28/cdc2 associated protein CLK	L29219	<0.1	0.3
Polo-like kinase	U01038	<0.1	<0.1
p53	AA143745	0.1	1
CDK4 inhibitor (p16-INK4)	L27211	5	ND
DNA binding protein transcription factors histones/repair			
KIAA0192/TRAP230	AF117755	3	1
Mel-18 hlog	AA477595	4	ND
OS-9	AB002806	2	1
DNA nucleotide exotransferase	AA744855	2	ND
Histone HUMGS00579	M37583	0.2	0.3
oriP binding protein (OBP-2)	L29606	<0.1	ND
RNA synthesis/ribosomes			
Clone 23689	AF035280	8	8
Ribosomal protein L37a	L22154	4	ND
Chemokines			
Interleukin-8	M28130	0.3	<0.1
GRO1α (melanoma growth-stimulating activity)	X57019	<0.1	<0.1
Tyrosine kinase receptors			
eck	NM_004431	3	3
axl	P30530	0.2	<0.1
Serine/threonine kinases			
Serum inducible kinase	O60679	7	2
Branched chain alpha keto acid dehydrogenase kinase	AF026548	4	1
Thymidylate kinase	L16991	0.5	0.3
Other receptors/integral membrane glycoproteins			
OX40	S76792	18	18
CXCR4	AF052572	13	6
Alpha 2 integrin	X17033	13	2
Podocalyxin-like protein	U97519	12	2
CD82	D28137	12	4
MUC18	M29277	5	1
MHC class I	M83191 D14343 M20022	3	3
gp130	M57230	3	1
T-cell receptor beta 2	X01411	3	2
MHC class II	L18885	2	ND
Protein zero related protein	R60084	6	6
Proteases/protease inhibitors			
Tissue factor pathway inhibitor-2	L27624	9	7
Aggrecanase (ADAMTS4)	NM_005099	18	2
Type IV collagenase (MMP9)	J05070	6	177

*Gene identified and confirmed by GeneCalling.
†GeneBank accession number of gene from which cDNA fragment was identified.
‡Refers to the median value of ratios for 24 hours/4 hours for the different cDNA fragments identified for a specific gene.
§TaqMan Ratio refers to the ratio of mRNA at 24 hours *versus* 4 hours as determined by Taqman (see Materials and Methods).
ND, not determined.

Table 3. Continued

Confirmed gene*	Accesion no.†	GeneCalling ratio‡	TaqMan ratio§
Type I collagenase (MMP1)	X05231	10	2
Cathepsin B	M14221	3	2
Plasminogen activator inhibitor-2	M31551	<0.1	<0.1
KIAA0188	d80010	3	2
Transporters/channels			
Glycoprotein-associated amino acid transporter LAT1	AJ130718	6	20
White protein Hlog	AF038175	3	0.5
Cytoskeleton/motility			
Brain ankyrin 2	H58696	2	1
Moesin	M69066	2	1
Myosin-IC	U14391	2	ND
Actin bundling protein	U09873	2	2
T-plastin	L05491	2	1
Dynein light chain	U32944	3	ND
Intermediate filaments			
Keratin K7	X03212	0.2	0.1
Extracellular matrix			
Laminin gamma 2 chain	U31201	12	49
Nidogen-2 (osteonidogen)	D86425	4	2
Type IV collagen	Y00706	5	1
S1-5 (EGF-containing fibulin-like extracellular matrix protein-1)	U03877	0.2	<0.1
p137	Z48042	3	1
Signal transduction			
Calmodulin	M27319	4	ND
Ras-related protein RAL-A	H94944	2	ND
TRAF1	U59863	2	1
MT-GRPE precursor	AA989480	2	ND
Nonreceptor tyrosine kinase	AF097738	4	1
Metabolic enzymes			
Z-crystallin/quinone reductase	AA316207	4	ND
S-adenosylmethionine synthase Hlog SAMS2	D11332	4	ND
OXA1 subunit of cytochrome oxidase	X80695	2	1
Antioxidant enzyme A0E37-2	U25182	0.3	0.5
Endoplasmic reticulum ATPase	W38423	2	ND
Posttranslational protein modification			
Peptidylglycine alpha amidating monooxygenase	AF035320	5	4
Ubiquitin 52 amino acid fusion protein	D28425	0.3	ND
Lipids and lipid turnover			
Phospholipase A_2 gamma	AF058921	11	21
Apolipoprotein E	AA087386	0.3	ND
Cyclooxygenase-2	M90010	0.1	<0.1
Coagulation system			
Tissue factor	J02931	0.5	0.1
Endosome/lysosome			
Lysosomal membrane sialoglycoprotein(CD36-2L)	D12676	2	ND
rab 5 interacting protein	S83365	2	ND
Clathrin components			
Clathrin heavy chain	AA100413	3	1
Clathrin assembly protein	U45976	3	ND
Unknown			
PMP41 Hlog (ALG2)	AA226371	6	2
Insulin-induced protein	W37284	5	ND
NK-4	M59807	3	ND
KIAA0726	AB018269	2	ND

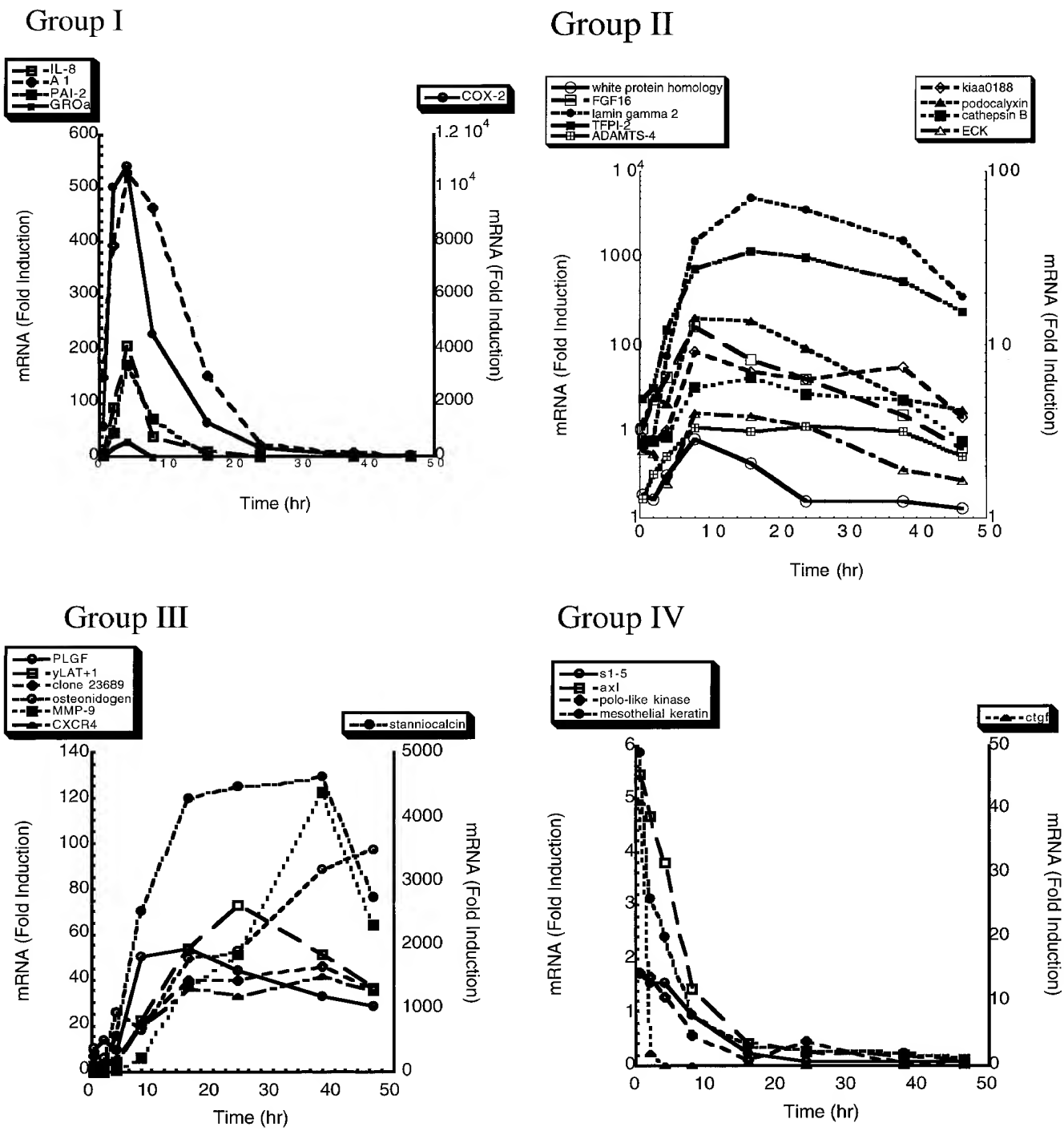


Figure 1. TaqMan analysis of the changes in gene expression of 26 genes identified by the GeneCalling analysis over the time period of 30 minutes to 46.5 hours. The changes in gene expression were grouped into four patterns, shown as Group 1 (rapid elevation in mRNA, peaking at 2–4 hours, then declining to baseline levels by 24 hours), Group II (more delayed elevation in mRNA, peaking at 8–12 hours, then declining to near-baseline levels by 46.5 hours), Group III (mRNA levels rising somewhat later than Group I or II, peaking at 12–46.5 hours, and remaining markedly above baseline levels at 46.5 hours), and Group IV (mRNA levels declining from the initial value observed at 30 minutes, and by 16–24 hours mRNA levels were below those obtained from quiescent HUVECs (see Materials and Methods)).

inhibit tumor angiogenesis in *in vivo* animal models[12] and in cancer patients.[13] Cathepsin B, a lysosomal cysteine protease, has been observed in many different tumor types and is highly expressed in tumor blood vessels as compared to normal vasculature.[14] One of the newest members of "disintegrin and metalloproteinase family members with thrombospondin motifs," ADAMTS-4 was identified as a differentially expressed gene in this study. Although relatively little information is available concerning ADAMTS-4, Tortorella et al[15] recently reported that this protein has aggrecanase activity and suggested that ADAMTS-4 played an important role in the turnover of the proteoglycan aggrecan in diseases such as osteoarthritis. Aggrecan has not previously been reported in vascu-

Table 4. Response of Modulated Genes to PPARγ Ligand 15d-PDJ_2

Confirmed gene*	Accesion no.†	TaqMan ratio‡	TaqMan ratio in 15d-PDJ_2-treated cells§
Hormones/growth factors			
Placental growth factor	X54936	5	0.4
FGF-16	AB009391	1	1
Stanniocalcin precursor	U25997	8	3
Cell cycle/apoptosis			
Bcl-2 related protein A1	L19597	1	1
Polo-like kinase	U01038	<0.1	15.5
RNA synthesis/ribosomes			
Clone 23689	AF035280	8	0.3
Chemokines			
Interleukin-8	M28130	<0.1	14.5
Tyrosine kinase receptors			
eck	NM_004431.1	3	2.3
axl	P30530	<0.1	15
Other receptors/integral membrane glycoproteins			
OX40	S76792	18	0.7
Podocalyxin-like protein	U97519	2	1
Proteases/protease inhibitors			
Tissue factor pathway inhibitor-2	L27624	7	1.3
Type IV collagenase (MMP9)	J05070	177	<0.1
Plasminogen activator inhibitor-2	M31551	<0.1	10.2
KIAA0188	d80010	2	0.5
Transporters/channels			
Glycoprotein-associated amino acid transporter LAT1	AJ130718	20	0.2
White protein Hlog	AF038175	0.5	2
Intermediate filaments			
Keratin K7	X03212	0.1	0.3
Extracellular matrix			
Laminin gamma 2 chain	U31201	49	4.9
Nidogen-2 (Osteonidogen)	D86425	2	1
Lipids and lipid turnover			
Phospholipase A_2 gamma	AF058921	21	0.8

A subset of genes identified as modulated in experiment were tested for response to PPARγ ligand 15d-PDJ2. Data are expressed as the ratio of expression observed at 24 hours/4 hours in collagen gel.

*Gene identified and confirmed by GeneCalling.

†GeneBank accession number of gene from which cDNA fragment was identified.

‡TaqMan ratio refers to the ratio of mRNA at 24 hours *versus* 4 hours.

§The ratio of mRNA at 24 hours *versus* 4 hours in the 15d-PDJ2-treated groups.

lar tissues, and we were unable to detect the expression of aggrecan mRNA in our endothelial cells under a variety of experimental conditions (data not shown). Intriguingly, however, two other members of the ADAM-TS family, METH-1 (human ADAMTS-1) and METH-2 (ADAMTS-8), were recently reported to be potent antiangiogenic agents.[16] The up-regulation of ADAMTS-4 during endothelial tube formation suggests a potential role of this enzyme in the metabolism of vascular proteoglycans, such as versican or other components of the basement membrane.

The mRNAs for the chemokines IL-8 and Gro-α are up-regulated early in the time course of endothelial differentiation into tubelike structures in the 3D gel environment (Figure 1A). These chemokines have previously been reported to have angiogenic activity.[17] mRNA levels for CXCR4, the chemokine receptor for SDF-1α,[18] increased by nearly 40-fold over a time period between 30 minutes and 16 hours, then remained elevated for the duration of the experiment (Figure 1C). Deletion studies have shown that both CXCR4 and SDF-1 null mice have defective formation of large blood vessels supplying the gastrointestinal tract.[19]

The mRNA level for the receptor "ECK" or epithelial tyrosine kinase (EphA2), a Eph receptor kinase family, increased modestly between 4 and 8 hours, and then slowly declined toward the levels observed at 30 minutes (Figure 1B). The ligand for ECK is a gene known as B61 (ephrin A1), initially identified as a tumor necrosis factor-α (TNFα)-induced gene in endothelial cells.[20] Antibodies to

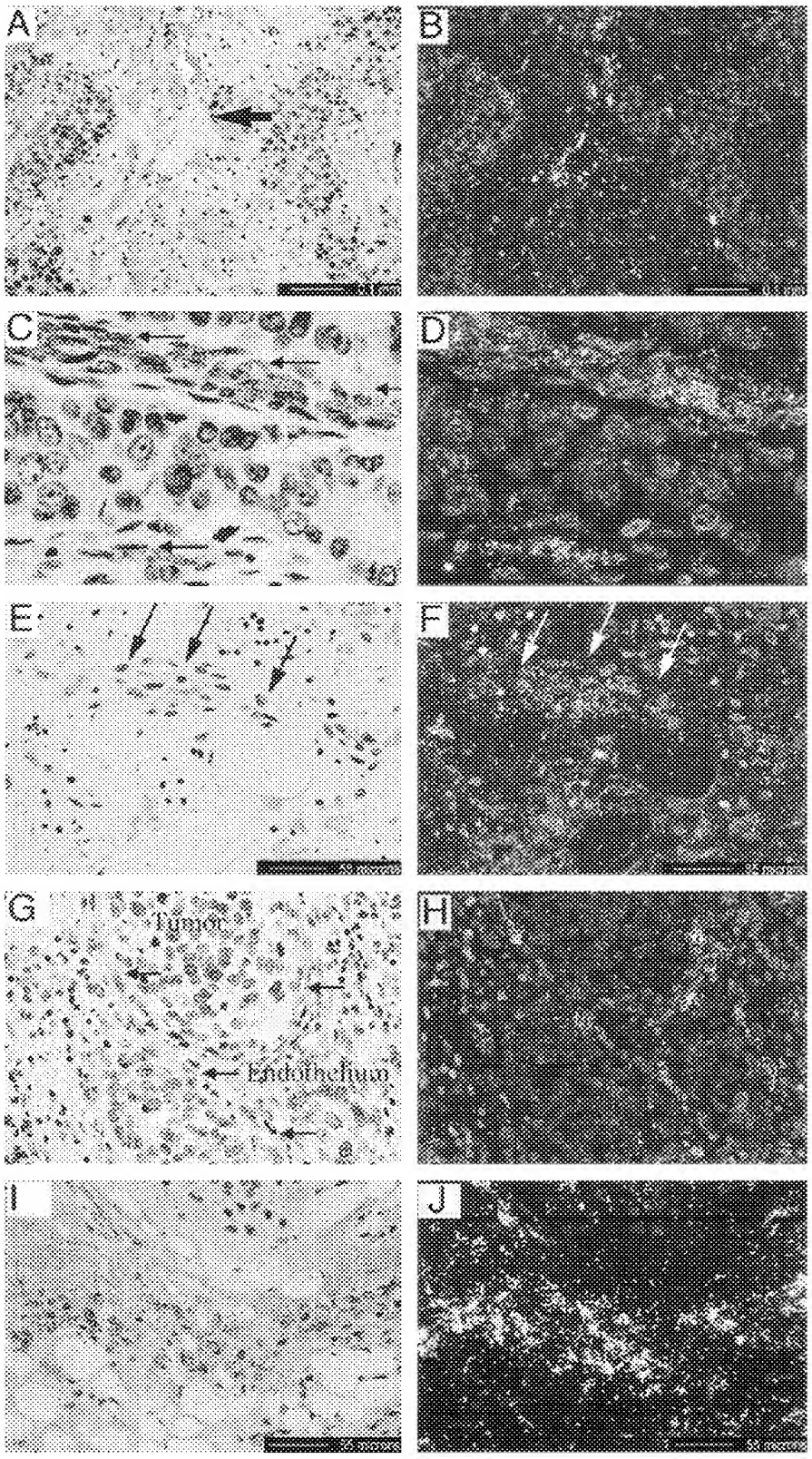


Figure 2. *In situ* hybridization demonstrating expression of genes identified from the differential expression analysis in the vasculature associated with tumors and with inflammatory disease. **A–D:** Hematoxylin-eosin (**A** and **C**) and *in situ* (**B** and **D**) hybridization demonstrating vascular expression (**arrows**) of stanniocalcin precursor mRNA in squamous cell carcinoma (**A** and **B**) and ductal mammary adenocarcinoma (**C** and **D**). **E** and **F:** Hematoxylin and eosin stain (**E**) and *in situ* (**F**) hybridization of osteonidogen mRNA in an arteriole (**arrows**) of inflamed appendix. **G** and **H:** Hematoxylin and eosin stain (**G**) and *in situ* (**H**) hybridization of podocalyxin expression in vessels surrounding lung squamous cell carcinoma (**arrows**). **I** and **J:** Hematoxylin and eosin stain (**I**) and *in situ* (**J**) hybridization of ADAMTS-4 expression adjacent to chondrosarcoma.

B61 block TNFα-induced angiogenesis in a corneal neovascularization assay.[21]

The mRNA for the laminin γ2 increased by almost 1000-fold between 0.5 and 24 hours (Figure 1B). Laminins are known to play key roles in angiogenesis[22] and are an important component of the basement membrane. Although the precise role of laminin in the differentiation process is unclear, laminin might be important for the establishment of the endothelial apical:basal polarity that may be a prerequisite for the formation of a vascular

lumen. The message levels for another basement membrane protein, osteonidogen (also known as Nidogen-2), increased throughout the duration of the HUVEC incubation in the 3D gels (Figure 1C). Nidogens can interact with laminin, collagen, and proteoglycans, and there are suggestions that this family of molecules may play some role in angiogenesis.[23]

In contrast to the genes discussed above, which have previously been implicated in various steps of angiogenesis, the study also identified a number of genes not previously associated with endothelial cell proliferation, differentiation, or angiogenesis.

The mRNA, designated KIAA0188, codes for a novel hypothetical protein. Domain analysis of the predicted amino acid sequence revealed a putative domain, aa 724–732, with homology to the consensus Kunitz-type serine protease inhibitor, and a domain, 455–467, with homology to the subtilase family of serine proteases, suggesting that this gene might code for a proform of a serine protease. KIA0188 mRNA levels increased modestly during the initial 8–10 hours of incubation in the gel, then declined to the initial levels (Figure 1B).

STC was first isolated from bony fishes,[24] where this glycoprotein is synthesized and secreted by the corpuscles of Stannius and regulates blood calcium levels through its inhibitory action on calcium ion uptake in the gill, a highly vascularized tissue.[24] Mammalian cDNAs encoding STC have been reported for the human and mouse and are highly homologous to those of the fish,[25] although the role of this protein in mammals is not known. mRNA levels for STC increased over 100-fold between 30 minutes and 16 hours and remained well above baseline levels out to 46.5 hours (Figure 1C). What role this gene plays in angiogenesis is unknown, but this observation suggests that further study in the context of angiogenesis is warranted.

The mRNA for FGF-16 demonstrated a biphasic profile, increasing substantially during the initial 8 hours and then declining for the remainder of the experiment (Figure 2B. This recent member of the FGF family of growth factors was originally cloned from human heart cDNA.[26] FGF-16 weakly stimulates NIH 3T3 fibroblast proliferation and but is a reasonably potent stimulus of primary rat oligodendrocyte proliferation.[27]

The mRNA levels of a number of membrane receptors were also increased over the time course of endothelial differentiation into tubelike structures, including the signaling component of the IL-6 receptor gp130, the TNFR-related protein OX40,[28] and the sialomucin, podocalyxin-like protein. There is no known role for any of these receptors in angiogenesis. Indeed, this study documents for the first time the expression of OX40 by endothelial cells. Previously this TNFR family member was thought to be restricted to cells of the lymphocyte lineage.[29] Increased protein expression of OX40 was also confirmed by fluorescence-activated cell sorter analysis (data not shown).

Podocalyxin-like protein, a well-known constituent of the endothelial plasma membrane,[30] was recently shown protein to function as an L-selectin receptor in inflamed lymph nodes,[31] suggesting a role in cell-cell interactions or adhesion.

The mRNA levels for the antiapoptotic bcl-2-related protein A1 were elevated at the early time points and then declined (Figure 1A). In the three-dimensional gel environments, HUVECs do not survive well in the absence of growth factors, and they cannot be rescued by supplementation with VEGF or bFGF.[1] However, PMA treatment will induce endothelial survival and tubule formation. The induction of A1 expression may thus be related to inhibition of apoptosis in the 3D gel environment.

The protein designated "white protein homolog" (also known as ATP-binding cassette (ABC) 8) is 84% identical to the *Drosophila* gene white protein, which codes for a transporter protein whose expression results in white eye color. Many members of the ABC family of proteins function as transporters or channels. The mRNA levels for this gene increased by ~10-fold between 0.5 and 8 hours (Figure 1B). There is little information relating to its expression or function in mammalian cells, although ESTs containing white protein sequence from a variety of tissue sources and tumors can be found in GenBank. The mRNA for y+LAT-1, a new member of a family of polytopic transmembrane proteins,[32] increased by about eightfold between 0.5 and 24 hours (Figure 1C). Little is known about the function of this permease, although it undoubtedly plays a role in amino acid transport and protein synthesis, two activities critical to altered endothelial protein expression.

Cyclooxygenase 2 (COX-2), a rate-limiting enzyme in the prostaglandin biosynthesis pathway, was detected in our experiment system. COX2 mRNA levels rose abruptly during the initial few hours of the experiment, then declined (Figure 1A). The transient expression we observed for COX2 is consistent with published reports proposing a role for COX2-regulated prostanoid responses after vascular injury.[33]

Xin et al[4] recently reported that agonists of PPARγ receptors specifically blocked endothelial tube formation *in vitro* and VEGF-driven angiogenesis *in vivo*. Furthermore, Xin et al[4] found that treatment of endothelial cells with the PPARγ ligand 15d-PGJ_2 inhibited the induction of kdr, flt-1, and uPA in a three-dimensional collagen gel model identical to the system used in the present investigation. The effects of 15d-PGJ_2 on mRNA levels for a number of genes identified in this study were therefore examined for the purpose of identifying genes modulated during tube formation specifically responsive to treatment with the PPARγ ligand. Those genes so identified might represent important targets for therapeutic intervention. As shown in Table 4, there were different classes of response to the PPARγ ligand (supermodulation, countermodulation, and no change in modulation), suggesting that the effect of treatment with 15d-PGJ_2 was not simply a general phenomenon. The selective and marked effects of 15d-PGJ_2 treatment on endothelial gene expression in the three-dimensional collagen system provides further support for the potential roles in angiogenesis of many of the genes identified in this study.

In summary, GeneCalling successfully identified 115 differentially expressed cDNA fragments corresponding

to 90 known genes from the study of collagen matrix-driven endothelial cell gene expression. In addition to the known genes identified, 80 fragments considered totally novel, or belonging to ESTs of unknown function, were identified in this study. The identity and expression of 67 of the known genes were confirmed by a second independent method (TaqMan). For the initial confirmations, we focused on membrane proteins and secreted proteins and only confirmed the identity of a few of the other cDNA fragments by this independent technique. However, in every instance the gene identified by the GeneCalling method was confirmed by TaqMan to be expressed by HUVECs in 3D gels, and the magnitude and direction of the changes in expression agreed reasonably well with the GeneCalling estimates. Because the method also identified a number of potentially new genes, we have not, at this time, pursued TaqMan confirmation of the remaining 23 genes, choosing to focus, instead, on the identification of new genes that might play a role in the process of endothelial differentiation into tubelike structures. Most importantly, the biological relevance of many of these newly identified "angiogenesis-associated" genes is strongly supported by the selective abrogation of their differential expression by the PPARγligand, 15d-PGJ$_2$, as well as by the *in situ* demonstration of selective expression of some of the genes at sites of new blood vessel formation. Although many of the identified genes have previously been associated with angiogenesis or tumor vasculature (eg, cathepsin B, MMP-9, PLGF, IL-8, GRO-α, CXCR4) or have reported roles or expression patterns consistent with a function in the differentiation process (A1, TFPI-2, laminin γ2), a number of genes identified in this study had never previously been associated with angiogenesis (stanniocalcin precursor, OX40, white protein homolog, the angiopoietin-2 homolog, ADAMTS-4, FGF16, KIAA0188), suggesting the need for the further evaluation of the potential biological roles of these genes in the process of new blood vessel formation

Acknowledgments

The authors wish to acknowledge the contributions made by the CuraGen Corporation Genomics Facility, the Genentech DNA synthesis facility, and Gretchen Frantz, Department of Pathology, Genentech. The authors gratefully acknowledge the helpful discussions of Dr. David Lowe, Genentech. The authors also acknowledge the University of Michigan (lung adenocarcinoma, breast tumor), National Disease Research Interchange (AMD eyes), and Western Infirmary, Glasgow (inflamed appendices, renal cell carcinoma), for the provision of human tissue samples used for the *in situ* studies.

References

1. Yang S, Graham J, Kahn J, Schwartz E, Gerritsen M: Differential roles for CD31 and VE-cadherin in formation of vascular tubes and lumens in three dimensional collagen gels. Am J Pathol 1999, 155:887–895
2. Shimkets RA, Lowe DG, Tai JT, Sehl P, Jin H, Yang R, Predki PF, Rothberg BE, Murtha MT, Roth ME, Shenoy SG, Windemuth A, Simpson JW, Simons JF, Daley MP, Gold SA, McKenna MP, Hillan K, Went GT, Rothberg JM: Gene expression analysis by transcript profiling coupled to a gene database query. Nat Biotechnol 1999, 17:798–803
3. Lu L, Gillett N: An optimized protocol for in situ hybridization using PCR generated ^{33}P-labeled riboprobes. Cell Vision 1994, 1:169–176
4. Xin X, Yang S, Kowalski J, Gerritsen ME: Peroxisome proliferator-activated receptor gamma ligands are potent inhibitors of angiogenesis in vitro and in vivo. J Biol Chem 1999, 274:9116–9121
5. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD: Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997, 277:55–60
6. Iino M, Foster DC, Kisiel W: Quantification and characterization of human endothelial cell-derived tissue factor pathway inhibitor-2. Arterioscler Thromb Vasc Biol 1998, 18:40–46
7. Petersen LC, Sprecher CA, Foster DC, Blumberg H, Hamamoto T, Kisiel W: Inhibitory properties of a novel human Kunitz-type protease inhibitor homologous to tissue factor pathway inhibitor. Biochemistry 1996, 35:266–272
8. Shinoda E, Yui Y, Hattori R, Tanaka M, Inoue R, Aoyama T, Takimoto Y, Mitsui Y, Miyahara K, Shizuta Y, Sasayama S: Tissue factor pathway inhibitor-2 is a novel mitogen for vascular smooth muscle cells. J Biol Chem 1999, 274:5379–5384
9. Rabbani SA: Metalloproteases and urokinase in angiogenesis and tumor progression. In Vivo 1998, 12:135–142
10. Werb Z, Vu TH, Rinkenberger JL, Coussens LM: Matrix-degrading proteases and angiogenesis during development and tumor formation. Apmis 1999, 107:11–18
11. Schnaper HW, Grant DS, Stetler-Stevenson WG, Fridman R, D'Orazi G, Murphy AN, Bird RE, Hoythya M, Fuerst TR, French DL, Quigley JP, Kleinman HK: Type IV collagenase(s) and TIMPs modulate endothelial cell morphogenesis in vitro. J Cell Physiol 1993, 156:235–246
12. Maekawa R, Maki H, Yoshida H, Hojo K, Tanaka H, Wada T, Uchida N, Takeda Y, Kasai H, Okamoto H, Tsuzuki H, Kambayashi Y, Watanabe F, Kawada K, Toda K, Ohtani M, Sugita K, Yoshioka T: Correlation of antiangiogenic and antitumor efficacy of N-biphenyl sulfonyl-phenylalanine hydroxiamic acid (BPHA), an orally active, selective matrix metalloproteinase inhibitor. Cancer Res 1999, 59: 1231–1235
13. Gradishar WJ: An overview of clinical trials involving inhibitors of angiogenesis and their mechanism of action. Invest New Drugs 1997, 15:49–59
14. Keppler D, Sameni M, Moin K, Mikkelsen T, Diglio CA, Sloane BF: Tumor progression and angiogenesis: cathepsin B and co. Biochem Cell Biol 1996, 74:799–810
15. Tortorella MD, Burn TC, Pratta MA, Abbaszade I, Hollis JM, Liu R, Rosenfeld SA, Copeland RA, Decicco CP, Wynn R, Rockwell A, Yang F, Duke JL, Solomon K, George H, Bruckner R, Nagase H, Itoh Y, Ellis DM, Ross H, Wiswall BH, Murphy K, Hillman MC Jr, Hollis GF, Newton RC, Magolda RL, Trzaskos JM, Arner EC: Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins. Science 1999, 284:1664–1666
16. Vazquez F, Hastings G, Ortega MA, Lane TF, Oikemus S, Lombardo M, Iruela-Arispe ML: METH-1, a human ortholog of ADAMTS-1, and METH-2 are members of a new family of proteins with angio-inhibitory activity. J Biol Chem 1999, 274:23349–23357
17. Strieter RM, Polverini PJ, Arenberg DA, Kunkel, SL: The role of CXC chemokines as regulators of angiogenesis. Shock 1995, 4:155–160
18. Bleul CC, Farzan M, Choe H, Parolin C, Clark-Lewis I, Sodroski J, Springer TA: The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature 1996, 382:829–833
19. Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, Kishimoto T, Nagasawa T: The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature 1998, 393:591–594
20. Holzman LB, Marks RM, Dixit VM: A novel immediate-early response gene of endothelium is induced by cytokines and encodes a secreted protein. Mol Cell Biol 1990, 10:5830–5838
21. Pandey A, Shao H, Marks RM, Polverini PJ, Dixit VM: Role of B61, the ligand for the Eck receptor tyrosine kinase, in TNF-alpha-induced angiogenesis. Science 1995, 268:567–569
22. Kubota Y, Kleinman HK, Martin GR, Lawley TJ: Role of laminin and basement membrane in the morphological differentiation of human

endothelial cells into capillary-like structures. J Cell Biol 1988, 107: 1589–1598
23. Nicosia RF, Bonanno E, Smith M, Yurchenco P: Modulation of angiogenesis in vitro by laminin-entactin complex. Dev Biol 1994, 164:197–206
24. Wagner G, Hampong M, Park C, Copp D: Purification, characterization, and bioassay of teleocalcin, a glycoprotein from salmon corpuscles of Stannius. Gen Comp Endocrinol 1986, 63:481–491
25. Chang AC, Janosi J, Hulsbeek M, de Jong D, Jeffrey KJ, Noble JR, Reddel RR: A novel human cDNA highly homologous to the fish hormone stanniocalcin. Mol Cell Endocrinol 1995, 112:241–247
26. Miyake A, Konishi M, Martin FH, Hernday NA, Ozaki K, Yamamoto S, Mikami T, Arakawa T, Itoh N: Structure and expression of a novel member, FGF-16, on the fibroblast growth factor family. Biochem Biophys Res Commun 1998, 243:148–152
27. Danilenko DM, Montestruque S, Philo JS, Li T, Hill D, Speakman J, Bahru M, Zhang M, Konishi M, Itoh N, Chirica M, Delaney J, Hernday N, Martin F, Hara S, Talvenheimo J, Narhi LO, Arakawa T: Recombinant rat fibroblast growth factor-16: structure and biological activity. Arch Biochem Biophys 1999, 361:34–46
28. Arch RH, Thompson CB: 4–1BB and Ox40 are members of a tumor necrosis factor (TNF)-nerve growth factor receptor subfamily that bind TNF receptor-associated factors and activate nuclear factor kappaB. Mol Cell Biol 1998, 18:558–565
29. Imura A, Hori T, Imada K, Ishikawa T, Tanaka Y, Maeda M, Imamura S, Uchiyama T: The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. J Exp Med 1996, 183:2185–2195
30. Stan RV, Roberts WG, Predescu D, Ihida K, Saucan L, Ghitescu L, Palade GE: Immunoisolation and partial characterization of endothelial plasmalemmal vesicles (caveolae). Mol Biol Cell 1997, 8:595–605
31. Sassetti C, Tangemann K, Singer MS, Kershaw DB, Rosen SD: Identification of podocalyxin-like protein as a high endothelial venule ligand for L-selectin: parallels to CD34. J Exp Med 1998, 187:1965–1975
32. Torrents D, Estevez R, Pineda M, Fernandez E, Lloberas J, Shi YB, Zorzano A, Palacin M: Identification and characterization of a membrane protein (y + L amino acid transporter-1) that associates with 4F2hc to encode the amino acid transport activity y + L. A candidate gene for lysinuric protein intolerance. J Biol Chem 1998, 273:32437–32445
33. Pritchard KA Jr, O'Banion MK, Miano JM, Vlasic N, Bhatia UG, Young DA, Stemerman MB: Induction of cyclooxygenase-2 in rat vascular smooth muscle cells *in vitro* and *in vivo*. J Biol Chem 1994, 269:8504–8509

ESTIS Document Delivery Service
University of Minnesota Libraries
Twin Cities Campus

Reference **11669.213AUWO DeMaster** # in order 1 **RQ # 61117**

Date In 5/17/2005 9:01:08 AM

BILL TO:

MARCIE RICHIE
MERCHANT & GOULD
3200 IDS CENTER
80 SOUTH EIGHTH STREET
MINNEAPOLIS MN
55402

612-371-5231 Phone
612.332.9081 Fax
0554-1339-8 Fedx
mrichie@merchant-gould.com Email

SHIP TO:

edemaster@merchant-gould.com

1 hour

Deadline

WEB SERVER

Library **MAGRATH**

Gerritsen, Mary E.; Wagner Graham F. Stanniocalcin:no longer just a fish tale. Vitamins & Hormones (2005) 70 105-35

Title: Vitamins and hormones. Current Vet
Author(s): Thimann, Kenneth Vivian,; 1904-
Publication: New York : Academic Press,
Year: 1943-
Description: Vol. 1-; v. :; ill. ;; 24 cm.
Standard No: ISSN: 0083-6729

Note

# of pages copied:

Copyright Fee $35.80

VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

Editor-in-Chief

GERALD LITWACK
Professor and Chair Emeritus
Department of Biochemistry and Molecular Pharmacology
Thomas Jefferson University Medical College
Philadelphia, Pennsylvania
Visiting Scholar
Department of Biological Chemistry
David Geffen School of Medicine of UCLA
Toluca Lake, California

VOLUME 70

ELSEVIER
ACADEMIC
PRESS

AMSTERDAM • BOSTON • HEIDELBERG • LONDON
NEW YORK • OXFORD • PARIS • SAN DIEGO
SAN FRANCISCO • SINGAPORE • SYDNEY • TOKYO

4

STANNIOCALCIN: NO LONGER JUST A FISH TALE

MARY E. GERRITSEN* AND GRAHAM F. WAGNER†

*Department of Molecular Pharmacology, Exelixis Inc., San Francisco, California 94083
†The Department of Physiology and Pharmacology, Faculty of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada N6A 5B8

Vitamins and Hormones
Volume 70

0083-6729/05 $35.00

Stanniocalcin was originally described as a hormone with calcitonin-like actions in fish. During the last decade, mammalian forms of stanniocalcin have been identified, and this discovery has led to important advances in our understanding of this enigmatic polypeptide hormone. This review briefly covers some early studies on stanniocalcin in fish and then provides a more in-depth look at some of the more intriguing, new aspects of its functions in mammals. The roles of stanniocalcin in renal function, metabolism, angiogenesis, pregnancy and lactation, bone formation, and neural protection are discussed, along with new information relating to its receptor-mediated sequestration and accumulation in target cell organelles.

I. GENERAL INTRODUCTION

Almost 10 years have passed since the first reported cloning of human stanniocalcin (STC). Up until that time, STC was considered to be an interesting but enigmatic polypeptide hormone with calcitonin-like actions in fish. Its discovery in humans has given way to steadily growing interest as to its possible roles in mammalian physiology. The range and scope of the articles that are being published at present is naturally quite broad as those in the field attempt to establish some sort of framework or unifying theme to STC's overall function. So far, this has not been possible and it is for this reason that the areas currently under investigation include topics as seemingly disparate as cellular chemotaxis, steroidogenesis, and respiration.

Mammalian STC has been the focus of a number of recent reviews (Chang *et al.*, 2003; Ishibashi and Imai, 2002), and when we were invited to write yet another on the topic, at first glance there did not appear to be much left to discuss. But after a period of reflection, we elected to reevaluate our own work and place it in context, wherever possible, with other work in the field. Indeed it seemed pointless to cover, once more, ground that was already well trodden.

For those with an interest in comparative endocrinology, our review commences with a brief historical overview on the discovery and functions of STC in fishes. With thanks to the archives from the University of Rostock in Germany, we have even managed to obtain a portrait of Professor Stannius, who initiated this entire field of research. We then focus on the mammalian field and, in particular, the more recent data relating to the effects of STC on cell proliferation and angiogenesis and what appears to be a unique type of cell signaling whereby STC is heavily sequestered by its target cells. It would be fair to say that the discovery process has at times been frustrating, especially when its role in fishes—calcium balance—seemed at first glance to be so "cut and dry." In truth, however, the more we learn about the mammalian system, the more we are being forced to reevaluate

many of our long-held notions concerning the fish hormone. Thus, while it is true that the discovery of STC in fish led to its subsequent discovery in mammals, what is now being revealed of its role in mammalian biology is in many cases leading to significant reappraisals of its roles and mechanisms of action in fish and, more recently, in invertebrates.

II. EARLY STUDIES ON THE DISCOVERY AND FUNCTION OF STANNIOCALCIN IN FISH

Stannius had been at the University of Rostock only 2 years (Fig. 1) when he pioneered the study of glands that are now referred to as the corpuscles of Stannius (CS). Unique to teleost and holostean fishes (bony fish), these

FIGURE 1. A portrait of Professor H. Stannius from the University of Rostock Archives where he was on staff from 1837 until 1873. Stannius died in 1883 at the age of 75.

glands had the appearance of small, cream-colored bodies, and because they were attached to the kidneys, Stannius naturally assumed they were fish adrenals. Hence, the title of his original article was *Concerning the Adrenal Glands of Bony Fishes* (Stannius, 1839). The term *corpuscles of Stannius* (CS) was coined in 1847 by Ecker (c.f. Vincent, 1898), and the belief that they were adrenals was to persist well into the 20th century. Light microscopy then revealed that CS cells had a high affinity for periodic acid Schiff reagent, suggesting that the products of the cells might in fact be glycosylated proteins (Krishnamurthy, 1976). Electron microscopy then revealed that the cells had the characteristics of polypeptide hormone-secreting cells, not of steroidogenic cells, which put to rest notions that they were in any way related to the adrenals of higher vertebrates (Ogawa, 1967).

Surgically ablating the glands, or stanniectomy (STX), was attempted as early as 1898, albeit to no effect (Vincent, 1898). Fontaine then had the presence of mind to look for possible changes in serum electrolyte levels, and he was the first to note that STX in the European eel resulted in frank hypercalcemia (Fontaine, 1964). Fenwick and colleagues followed up on this discovery by showing that the rate of gill calcium transport from the water into the extracellular compartment was markedly increased following STX and could be corrected by the infusion of CS glandular extracts. This finding definitively pinpointed the gills as the cause of the hypercalcemia and led to the conclusion, that still holds today, that the active principle elaborated by the glands was an inhibitor of gill calcium transport (Fenwick and So, 1974; So and Fenwick, 1979).

Purification of the active principle, now known as STC, revealed it to be a 50-kDa disulfide-linked dimer of identical 25-kDa subunits (Hulova and Kawauchi, 1999; Lafeber *et al.*, 1988; Wagner *et al.*, 1986). The hormone was also shown to be glycosylated, and Blast searches have revealed that its amino acid sequence is unique. The hormone also underwent a number of name changes (hypocalcin, teleocalcin) before those working in the field finally settled on stanniocalcin, in honor of Stannius's discovery. In fish, STC is released into the bloodstream in response to rising serum calcium levels, the stimulus being mediated through calcium-sensing receptors (Radman *et al.*, 2002). The released hormone then acts on both gill and gut epithelial cells to reduce the uptake of calcium. Within the kidney, STC increases the tubular reabsorption of phosphate, an effect that is presumably intended to chelate and remove the excess calcium from the extracellular compartment. The net effect of these actions is a restoration of serum calcium levels.

Interestingly, whereas STC was originally believed to be synthesized only by CS cells, we now know that the gene is expressed in most tissues though at much lower levels. Furthermore, the gene products appear to be somewhat different. For instance, a more heavily glycosylated form of STC is produced in gonadal tissue from the same RNA transcript. Presumably, these STC

variants function only locally. However, the fact that STC is produced in significant amounts by other tissues could serve to explain why fish are able to mount a complete recovery from the effects of STX 4 to 8 weeks after surgery (McCudden *et al.*, 2001a). For those with a specific interest in comparative endocrinology, there are numerous extant reviews dealing with various facets of the history and biology of STC in fishes (Flik, 1990; Krishnamurthy, 1976; Wagner, 1993, 1994; Wendelaar Bonga and Pang, 1986, 1991).

III. THE DISCOVERY OF MAMMALIAN STANNIOCALCIN

Following the discovery of the CS glands and even after the characterization of the fish hormone, the prevailing opinion was still that both the glands and the hormone were unique to fish. The general view was that because fish were not on the main line of vertebrate evolution, they had undoubtedly evolved genes and structures that simply did not find their way into higher vertebrates. In addition, mammals already had a full complement of hormones for regulating mineral balance, and there seemed to be little room for yet another hormone. At the same time, however, it was well known that the majority of fish peptide hormones had been retained throughout mammalian evolution and, so, why not STC? Our search for a mammalian homologue was driven largely by this fact as well as by compelling data we had begun gathering at the time that pointed to the existence of STC-related proteins in annelid worms. If STC was present in such early invertebrates, was it not highly unlikely to be found in just one of the five vertebrate classes?

If STC was indeed present in mammals, the question then became where was the best place to look? At the time, the CS glands were still considered to be the sole source of STC in fish and because they were known to be derived from renal tubular cells (Kaneko *et al.*, 1992), the kidney appeared to be the logical first choice. Conventional library screenings with fish nucleotide probes proved to be entirely unsuccessful, and we had just managed to identify several STC-immunoreactive proteins in human kidney extracts (Wagner *et al.*, 1995) when the field made a quantum leap forward with the cloning of human STC by two separate laboratories. At the Children's Medical Research Institute in Australia, Reddel's group was using differential display to identify genes involved in cell proliferation. A transcript with high homology to fish STC that was down-regulated following transformation with SV40 subsequently proved to be human STC (Chang *et al.*, 1995). At much the same time, scientists at Human Genome Sciences in Rockville, Maryland, were characterizing a full-length human STC cDNA that they had isolated from human embryonic lung using the expressed sequence tag strategy pioneered by Craig Venter, who was then at The Institute for

Genomic Research, also in Rockville, Maryland. Human Genome Sciences also produced the first generation of recombinant human STC (rhSTC) using a bacterial expression system, and were able to demonstrate its regulatory effects on calcium and phosphate transporters in mammalian and fish systems (Olsen *et al.*, 1996). The cloning of human STC made possible the development of all the tools and reagents that are now currently in use by laboratories all over the world and, as such, has to be viewed as the singular achievement in the field.

A. STRUCTURAL FEATURES OF MAMMALIAN STANNIOCALCIN

Human STC (hSTC) proved to be surprisingly homologous to the fish hormone. The primary translation product is 247 residues in length versus 256 in fish. The level of sequence similarity to salmon STC is 92% in the first 204 residues and 73% overall (Chang *et al.*, 1996; Olsen *et al.*, 1996). The C terminus is highly divergent, however, as it is in fishes, indicating that biological activity likely resides in the core and N-terminal domains. Perhaps the most notable of its many features are the 11 cysteine (Cys) residues (Fig. 2) that are completely conserved in mammals and fish. The unpaired Cys at position 170 (169 in fish) allows for the formation of a single interchain disulfide linkage and the creation of the 50-kDa homodimer (Zhang *et al.*, 1998). The importance of the dimer has been called into question, however, by the recent discovery of exclusively monomeric forms of the hormone in more primitive fish, in which Cys_{169} has been replaced by an arginine with no apparent ill effects on the maintenance of calcium balance (Amemiya *et al.*, 2002). Indeed, as both of the STC-related proteins recently identified in annelids appear to be monomers as well, the dimer may only have arisen in vertebrates (Tanega *et al.*, 2004). The N-linked glycosylation consensus sequence has also been conserved in mammals and, as in fish, is occupied by a carbohydrate moiety (Niu *et al.*, 2000; Zhang *et al.*, 1998).

Unexpectedly, the mammalian gene proved to be much more widely expressed than the fish hormone, which at the time was believed to be confined to the CS glands. Northern blot analysis revealed that transcript was readily detectable in tissues such as kidney, adrenal, heart muscle, lung, thymus, and skeletal muscle, to name only some. By far the highest levels of expression, however, were seen in adult ovaries, which was in stark contrast to the testes where transcript was barely detectable. There also proved to be two transcripts in mammals (2 and 4 kb) in comparison to one 2-kb transcript in fish (Varghese *et al.*, 1998), the significance of which still remains to be established.

The broad-based tissue pattern of expression in mammals prompted of course an immediate reexamination of *STC* gene expression in fish and revealed, as described in Section II, that the fish gene was just as widely

```
Mouse STC MLQNSAVILALVISAAAAHEAEQNDSVSPRKSRVAAQNSAEVVRCLNSAL  50
Rat   STC MLQNSAVILALVISAAAAHEAEQNDSVSPRKSRVAAQNSAEVVRCLNSAL  50
Human STC MLQNSAVLLVLVISASATHEAEQNDSVSPRKSRVAAQNSAEVVRCLNSAL  50
Bovine STC MLQNSAVILVLVISASATHEAEQNDSVSLRKSRVAAQNSAEVIRCLNSAL  50
Salmon STC MLAKFGLCAVFLVLGTAATFDTDPEEASPRRARFSSNSPSDVARCLNGAL  50

Mouse STC QVGCGAFACLENSTCDTDGMYDICKSFLYSAAKFDTQGKAFVKESLKCIA  100
Rat   STC QVGCGAFACLENSTCDTDGMYDICKSFLYSAAKFDTQGKAFVKESLKCIA  100
Human STC QVGCGAFACLENSTCDTDGMYDICKSFLYSAAKFDTQGKAFVKESLKCIA  100
Bovine STC QVGCGAFACLENSTCDTDGMYDICKSFLYSAAKFDTQGKAFVKESLKCIA  100
Salmon STC AVGCGTFACLENSTCDTDGMHDICQLFFHTAATFNTQGKTFVKESLRCIA  100

Mouse STC NGITSKVFLAIRRCSTFQRMIAEVQEDCYSKLNVCSIAKRNPEAITEVIQ  150
Rat   STC NGITSKVFLAIRRCSTFQRMIAEVQEDCYSKLNVCSIAKRNPEAITEVIQ  150
Human STC NGVTSKVFLAIRRCSTFQRMIAEVQEECYSKLNVCSIAKRNPEAITEVVQ  150
Bovine STC NGVTSKVFLAIRRCSTFQRMIAEVQEECYTKLNVCSVAKRNPEAITEVVQ  150
Salmon STC NGVTSKVFQTIRRCGVFQRMISEVQEECYSRLDICGVARSNPEAIGEVVQ  150

Mouse STC LPNHFSNRYYNRLVRSLLECDEDTVSTIRDSLMEKIGPNMASLFHILQTD  200
Rat   STC LPNHFSNRYYNRLVRSLLECDEDTVSTIRDSLMEKIGPNMASLFHILQTD  200
Human STC LPNHFSNRYYNRLVRSLLECDEDTVSTIRDSLMEKIGPNMASLFHILQTD  200
Bovine STC LPNHFSNRYYNRLVRSLLDCDEDTVSTIRDSLMEKIGPNMASLFHILQTD  200
Salmon STC VPAHFPNRYYSTLLQSLLACDEETVAVVRAGLVARLGPDMETLFQLLQNK  200

Mouse STC HCAQTHPRADFN------RRRTNEPQKLKVLLRNLRGEGDSPSHIKRTSQE  245
Rat   STC HCAQTHPRADFN------RRRTNEPQKLKVLLRNLRGEGDSPSHIKRTSQE  245
Human STC HCAQTHPRADFN------RRRTNEPQKLKVLLRNLRGEEDSPSHIKRTSHE  245
Bovine STC HCAHTQQRADFN------RRRANEPQKLKVLLRNLRGEVASPSHIKRTSQE  245
Salmon STC HCPQGSNQGPNSAPAGWRWPMGSPPSFKIQP-SMRGRDPTHLFARKRSVE  249

Mouse STC SA-----  247
Rat   STC NA-----  247
Human STC SA-----  247
Bovine STC SA-----  247
Salmon STC ALERVME  256
```

FIGURE 2. Comparison of the mammalian and fish stanniocalcins. The alignment was performed by the ClustalW program. Courtesy of European Bioinformation Institute (2004). The box sequence (NST) represents the glycosylation consensus sequence.

expressed (McCudden *et al.*, 2001b). A major difference between mammals and fish emerged, however, with the development of immunoassays for the measurement of serum hormone levels in mammals. In contrast to the fish in which STC circulates continuously for the maintenance of serum calcium levels, mammalian serum turned out to contain little if any STC immunoreactivity. This has led us to conclude that in the majority of mammalian organ systems, endogenous STC production is intended for purely local signaling pathways (Niu *et al.*, 2000). Indeed, the only instances in which measurable levels of STC immunoreactivity have been observed in mammalian serum are in females and, even then, only during pregnancy and lactation (Section IV.D).

The gene-encoding hSTC is on the short arm of chromosome 8 (8p11.2p21), and the genomic structures of the human and mouse genes

have both been elucidated. Each contains four exons, spanning 13 kb of DNA (Varghese *et al.*, 1998). In contrast, the salmon gene contains five exons spanning just 4 kb; mammalian exon 3 is split between exons 3 and 4 in fish, and the fish gene has less intronic DNA (McCudden *et al.*, 2001a).

The human gene also has 19 CAG trinucleotide repeats—often associated with instability (Lenzmeier and Freudenreich, 2003)—within 102 nucleotides of the transcription start site. The role of the repeats is unknown, but they could be potential sites of transcriptional control.

Stanniocalcin-related protein (STCrp), or STC2, is the product of a second related gene and shares 34% overall homology with STC, which is sometimes now referred to as STC1 (Chang and Reddel, 1998; DiMattia *et al.*, 1998; Ishibashi *et al.*, 1998; Moore *et al.*, 1999). The tissue pattern of STC2 gene expression is markedly different, however, and there is much less available information concerning its overall function. A comprehensive review on the comparative aspects of STC and STC2 structure and expression patterns and their potential roles in cancer has been published (Chang *et al.*, 2003).

B. BIOLOGICAL ACTIVITIES OF MAMMALIAN STANNIOCALCIN

The cloning of human STC led to the production of recombinant protein, first by Human Genome Sciences (Zhang *et al.*, 1998) and subsequently by other groups, as well as the production of antibodies for immunoassay development. As already mentioned, the first generation of recombinant human STC (rhSTC) was expressed in bacteria and yet was even bioactive in fish (Olsen *et al.*, 1996). More importantly, in homologous mammalian organ systems (kidney and intestine), bacterially expressed hSTC had the same regulatory effects as the fish hormone (Lu *et al.*, 1994; Sundell *et al.*, 1992) on the transepithelial movements of calcium and phosphate. In unconscious male rats, bolus injections of bacteria-derived rhSTC have been shown to stimulate the tubular reabsorption of phosphate, with no attendant effects on the renal handling of sodium, potassium and calcium, glomerular filtration rate, or renal blood flow. The phosphate effect proved to be due to a doubling in NaPT2 cotransporter activity (Olsen *et al.*, 1996; Wagner *et al.*, 1997). Similarly, in rodents and swine, the same bacterially expressed hormone significantly reduced the intestinal uptake of calcium while enhancing that of phosphate (Madsen *et al.*, 1998). In most respects, the effects mirror those of STC in homologous fish organ systems.

The expression of STC in developing bone and the critical importance of this tissue to mineral homeostasis have prompted several investigators to evaluate its patterns of expression and the role of the hormone in bone mineralization. In axial skeleton, STC gene is transiently expressed in a rostral–caudal fashion during vertebral development. Gene expression is

confined to intervertebral disc mesenchyme cells, and the ligand is targeted to vertebral hypertrophic and prehypertrophic chondrocytes. The pattern of expression in the appendicular skeleton is equally striking. Early in development, STC gene expression defines the initial lengths of bone primordia. The gene is expressed in mesenchyme cells at either ends of precartilaginous condensations defining future long bones, and the ligand is targeted to the chondroblasts. Later during joint formation, STC is highly expressed in the interzone cells defining all future joints. Following cavitation (joint formation), STC gene expression is greatest in those perichondrocytes that are found lining the joints, whereas the underlying resting, proliferative, and prehypertrophic chondrocytes appear to be targeted by the hormone both during and after cavitation. Hence, STC's pattern of expression indicates its roles in early skeletal patterning as well as joint formation (Stasko and Wagner, 2001a).

Yoshiko *et al.* (2002) have demonstrated that rhSTC stimulates bone mineralization by increasing phosphate uptake into osteoblasts, the mechanism involving an up-regulation in *Pit1*, a sodium-dependent phosphate transporter. In rat calvarial cultures, the same group found that STC overexpression (additions of rhSTC) and underexpression (antisense oligonucleotides) accelerated and retarded, respectively, osteogenic development. During nodule formation, rhSTC increased both sodium-dependent phosphate uptake and *Pit1* gene expression, but this was not the case in the more primitive progenitor stages (Yoshiko *et al.*, 2003).

Additional functions of STC have been proposed based on expression profiles under certain physiological or pathophysiological states. For example, because STC is abundant in human and mouse neuronal cells (Stasko and Wagner, 2001a; Zhang *et al.*, 2000), it has been suggested that the hormone plays a role in axonogenesis. Indeed, the STC gene is up-regulated markedly in terminally differentiating mouse neuroblastoma cells, and key markers of the axonal phenotype fail to materialize in cells treated with antisense STC oligonucleotides (Wong *et al.*, 2002; Yeung *et al.*, 2003). Others have noted a strong correlation between STC expression and hypoxic damage to pyramidal cells of the cerebral cortex as well as altered expression patterns around brain infarcts, leading to the supposition that STC may protect neuronal cells against hypoxic damage and Ca^{2+}-mediated cell death (Zhang *et al.*, 2000). Similarly, the transfection of neural crest-derived human neuronal cells (Paju line) with the full-length hSTC cDNA increased their resistance to hypoxic stress and high intracellular calcium levels. Interestingly, the STC effect was mediated in part by an increase in cellular phosphate transport, presumably as a means of chelating the excess calcium (Zhang *et al.*, 2000).

Several laboratories have reported the up-regulation of STC in angiogenic endothelial cells, either using *in vitro* assays (e.g., tubulogenesis, treatment with growth factors) or in tumor vasculature *in vivo* (Bell *et al.*, 2001; Gerritsen *et al.*, 2002; Kahn *et al.*, 2000; Liu *et al.*, 2003; Wary *et al.*, 2003).

Zlot *et al.* (2003) evaluated several potential roles of STC in endothelial cells. They were unable to demonstrate any effect of rhSTC on endothelial cell proliferation, tube formation, or apoptosis; however, rhSTC was shown to inhibit hepatocyte growth factor (HGF)-induced endothelial cell migration. The effect of rhSTC appeared to be selective for HGF because the chemotactic response to vascular endothelial growth factor and basic fibroblast growth factor were unaffected by STC cotreatment. STC did not block HGF from interacting with its receptor, *c-met*, since tyrosine phosphorylation of the receptor following HGF addition was not inhibited by hormone addition. However, rhSTC did reduce HGF-induced phosphorylation of focal adhesion kinase, suggesting that its inhibitory effects were downstream of HGF receptor activation.

The STC gene is also highly expressed in the thymus and spleen, suggesting a possible role in the immune and inflammatory responses. Kanellis *et al.* (2004) found that rhSTC reduced spontaneous chemokinesis and the chemotactic response of monocytic cells to the chemokines MCP-1 and SDF-1α. This effect appeared to be mediated, at least in part, by a reduction in the chemokine-induced $[Ca^{2+}]_i$ signal although the concentrations of rhSTC required to maximally inhibit the calcium response (10 nM) were greater than those required to inhibit chemotaxis (2 nM).

STC is also highly expressed in heart and skeletal muscle, which suggests its possible roles in cardiac and skeletal myocyte function. Sheikh-Hamad *et al.* (2003) examined the expression and localization patterns of STC mRNA and protein in cardiac tissue from patients with advanced heart failure before and after unloading, using a left ventricular assist device (LVAD), and compared this data with normal heart tissue (Sheikh-Hamad *et al.*, 2003). They found that the STC protein was markedly up-regulated in cardiomyocytes and arterial walls of the failing heart and, furthermore, that STC expression was reduced after LVAD treatment. The addition of rhSTC to cultured rat cardiomyocytes reduced the beating rate and reduced the rise in Ca^{2+} associated with each contraction. As a possible mechanism of action, whole cell patch clamp studies demonstrated that rhSTC was capable of reversibly inhibiting transmembrane L-channel calcium currents. Intriguingly, the common denominator for many of the studies outlined in the latter paragraphs is how often STC's effects appear to be mediated by alterations in the transmembrane fluxes of either calcium or phosphate.

C. STC TRANSGENICS

Experiments involving mouse transgenesis have provided additional insights regarding the potential roles of STC in mammals. Two transgenic approaches have been used. Filvaroff *et al.* (2002) placed hSTC under the control of a muscle-specific promoter, whereas DiMattia and colleagues expressed the hormone more ubiquitously using a transgene driven by the

metallothionein 1 promoter (Varghese *et al.*, 2002). In both cases, the STC overexpressing mice exhibited a growth retardation phenotype (30–50%) that persisted after weaning. Moreover, when wild-type (WT) pups were nursed by transgenic (Tg) mothers, the WT pups exhibited the same phenotype. This finding could indicate changes in either nursing behavior, milk production rates, or, perhaps, milk composition. The dwarf phenotype could not be attributed to differences in serum calcitonin, IGF binding protein, growth hormone, or T4 levels, which were all similar in WT and Tg animals. Both the axial and appendicular skeletons were shorter in Tg mice, but they had the same number of vertebrae and histologically normal femoral growth plates. Radiographs of the skull bones showed that they had altered suture morphology and decreased cellular extravasation through the center of the parietal bones, which indicated decreased osteoclast activity. The quality of bone formed was examined by histology and μCT scans; although von Kossa staining did not reveal striking differences in the cortical and trabecular zones, quantitative μCT scanning revealed that cortical bone volume and total volume (bone and bone marrow) were both reduced in Tg mice.

Furthermore, both groups found elevated serum calcium levels in at least some of the Tg lines (Filvaroff *et al.*, 2002; Varghese *et al.*, 2002) and, in some cases, elevated levels of serum inorganic phosphate (Varghese *et al.*, 2002). Measurements of osteoblast activity were also lower in the Tg mice, and, in addition to being smaller, the Tg mice were remarkably lean (Filvaroff *et al.*, 2002). The latter observation prompted additional studies on metabolism, whereby daily food intake, oxygen consumption, and measures of glucose tolerance were each evaluated in the Tg and WT lines. Interestingly, the Tg mice ate 32.2% more, weighed 36% less, and consumed 13.8% more oxygen than their WT littermates. They also exhibited faster rates of glucose clearance. Collectively, the data were indicative of STC overexpression resulting in higher rates of metabolism and possibly a developmental phenotype (Filvaroff *et al.*, 2002).

Filvaroff *et al.* (2002) also noted significant differences in the skeletal muscle of STC transgenic mice. All of the muscles of the STC Tg mice weighed significantly less than those of age-matched control mice (in contrast, organs such as the kidney weighed more in Tg mice than age-matched controls when analyzed as a percentage of body weight). At the ultrastructural level, these disproportionately smaller skeletal muscles had enlarged myocyte mitochondria. To evaluate muscle function, the contractile responses of muscles to a repetitive pulsatile electrical stimulation were compared in both groups. Although the maximal twitch response was not different in Tg relative to WT mice, the stable twitch response was significantly attenuated, potentially reflecting a reduction in muscle function in the STC overexpressing mice. Because endothelial differentiation has been associated with changes in STC expression, baseline vascular density and angiogenic responses were also measured following femoral artery ligation.

Baseline vascular density was determined using a radiolabelled CD31 technique, which is a measure of endothelial cell content. Interestingly, the Tg mice had higher vascular densities in all organs, including skeletal muscle, in comparison to age-matched WT littermates. Histologically, however, there were no obvious differences in tissue vasculature, suggesting that vascular architecture was normal.

Femoral artery ligation produces pronounced ischemia in the lower limb and is followed by a marked increase in the development of new blood vessels (angiogenesis) that usually peaks between 5 to 7 days after ligation. When this procedure was conducted on Tg and WT mice, the Tg animals demonstrated a robust angiogenic response upon ligation and subsequently attained a higher vascular density than their WT littermates. Whereas WT animals recovered most of their muscle function, the Tg mice were noticeably impaired 7 days postligation (Filvaroff *et al.*, 2002). It is possible that myocyte mitochondrial hypertrophy is partly responsible for this impaired recovery. The hypothesis is particularly attractive in view of the fact that mitochondria are now known to be heavily targeted by STC (Section III.D).

Reproductive potential and/or fecundity were also impaired in *STC* overexpressing mice, evident by the fact that litter sizes were significantly reduced (Varghese *et al.*, 2002). Because the ovaries are known to have the highest levels of STC in the adult rodent (Deol *et al.*, 2000; Varghese *et al.*, 1998), the findings were indicative of a role for STC during pregnancy.

D. STC PHARMACOKINETICS AND PUTATIVE BLOOD-BORNE BINDING PROTEINS

Human Genome Sciences was also first off the mark in developing second generation forms of rhSTC, this time in baculovirus and mammalian expression systems. In spite of minor differences in N-terminal processing, both recombinant proteins have proven to be equipotent in dose-response bioassays and in their clearance kinetics (Niu *et al.*, 2000; Zhang *et al.*, 1998). More importantly, pharmacokinetics have helped us understand why STC cannot be measured in mammalian serum under most circumstances using conventional immunoassays and, at the same time, have provided intriguing insights as to STC's fate once it enters the circulation.

Initially, the pharmacokinetics were carried out using the standard technique, whereby unconscious rats received bolus injections of ^{125}I-labelled STC tracer via the jugular vein, and blood was sampled over time from the femoral artery. Under these conditions, rhSTC behaved like most other mammalian polypeptide hormones. In males and females, rhSTC distributed in the extracellular compartment within a minute ($t1/2_{\alpha} = 0.98$ min) and cleared from the circulation within an hour ($t1/2_{\alpha} = 60.7$ min). Comparatively speaking, the results were unremarkable (Niu *et al.*, 2000).

More interesting results were obtained, however, when the study was repeated using the same hormone preparation, this time in the nonradiolabelled state. Furthermore, because the animals were now being injected with "cold" hormone, clearance was monitored instead with an STC immunoassay. Under these conditions, STC exhibited an altogether different pattern of clearance. The cold hormone distributed in the extracellular compartment just like the tracer, but it had no discernable second phase, or $t1/2_{\beta}$, during which STC was supposedly being cleared from the circulation (Niu *et al.*, 2000) (Fig. 3). By all accounts, it appeared that the cold hormone was being rendered nonimmunoreactive while it was distributing in the extracellular compartment. The hormone was obviously still there in the serum, but the immunoassay could not detect it.

Findings such as these can be interpreted in a number of different ways. The simplest explanation is that an enzyme in the vascular compartment is modifying STC and, in the process, rendering it nonimmunoreactive and perhaps biologically inactive. The latter certainly occurs in fish where there is virtually no correlation between plasma levels of bioactive STC and those

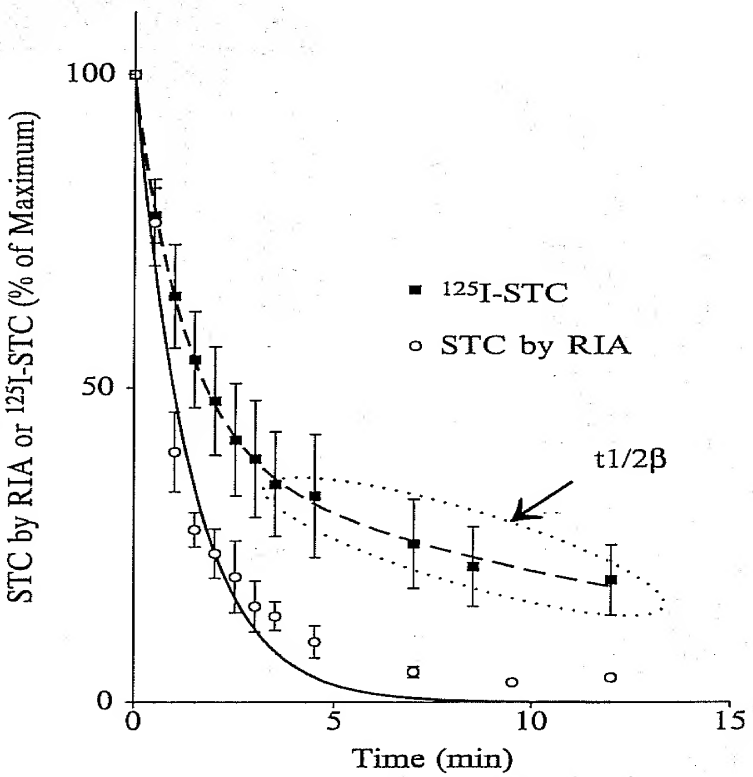


FIGURE 3. Clearance kinetics of STC in unconscious adult male rats. Animals were given bolus injections of ^{125}I-hSTC (20×10^6 cpm; 100 ng in total) or 100 μg of unlabelled (cold) hormone via the jugular vein. Heparinized blood samples were taken from the femoral artery every 0.5 min for the first 4.5 min and every 2.5 min thereafter. The disappearance of tracer was monitored by gamma-counting of the trichloroacetic acid-precipitable plasma radioactivity. The disappearance of cold hormone was monitored by immunoassay. Note that in comparison to ^{125}I-hSTC, the cold hormone had no $t1/2_{\beta}$. Adapted from Niu *et al.* (2000).

measured by immunoassay (Wagner *et al.*, 1993). Alternatively, and there is better evidence for this, the blood could contain a binding protein that effectively masks all antibody epitopes on the hormone and, in doing so, renders it undetectable by immunoassay. Such a binding protein could be soluble and freely circulating in the bloodstream, as is the case for many of the IGF binding proteins (Murphy, 2003), or it could be tethered to endothelial cells as in the case of the ANF clearance receptors (Silberbach and Roberts, 2001).

Evidence for a soluble, blood-borne interfering substance already exists in mammals although the precise nature of the interference (enzymatic or binding activity) has not been ascertained. When rhSTC is added to fresh mammalian serum and then subsequently measured by immunoassay, there is substantially less than complete recovery (60–80%) of the added hormone (Niu *et al.*, 2000). Such findings are clearly indicative of interfering substances within the serum, and they could also explain why under most circumstances STC cannot be reliably measured in mammalian serum using conventional immunoassays (Niu *et al.*, 2000). Evidence of blood-borne binding activity has been obtained in the red blood cell fraction and also through pharmacokinetics. Following the injection of ^{125}I-STC into rats as already described, the red blood cell fraction reversibly binds ^{125}I-STC in direct proportion to the levels of tracer in the plasma, such that the disappearance curves for ^{125}I-STC from the red blood cell fraction and from blood plasma are virtually superimposable (Niu *et al.*, 2000). Of interest from a physiological standpoint, the binding of STC to red blood cells could be a mechanism for systemic hormone delivery to targets such as liver, which exhibit little if any evidence of gene activity and yet contains substantial levels of STC immunoreactivity.

IV. THE SEQUESTERING HYPOTHESIS

In the case of most if not all hormones, if one were to map the tissue distribution of the ligand by immunocytochemistry and its transcript by *in situ* hybridization, the two would colocalize together at the sites of synthesis. Moreover, there would be little or no ligand detected in nearby target cells because it is generally recognized that most ligands are internalized and destroyed following receptor binding. This is not the case for STC in mammals. A large body of evidence now indicates that the STC ligand accumulates in its target cells to such an extent that they become heavily stained following conventional immunocytochemistry, giving the mistaken impression that they are, in fact, sites of STC production. Evidence for this has been found in a number of mammalian species.

Kidney is a tissue that has been examined in considerable detail in the mouse, staged embryos, neonates, and adults. At all stages in life history,

STC gene expression is confined to collecting duct principal cells (Deol *et al.*, 2000; Wong *et al.*, 1998), and, as expected, principal cells also contain high levels of STC immunoreactivity (STCir). Equally high levels of STCir, however, can be found in the cells of more upstream nephron segments that have no discernable gene activity. These include the distal convoluted tubules, cortical and medullary thick ascending limbs, and the S2/S3 segments of the proximal tubule (Haddad *et al.*, 1996).

A similar pattern is seen in mouse ovary. Although ovarian STC gene expression is confined to the androgen-producing theca and interstitial cells, the levels of STCir are often higher in nearby oocytes and corpus luteal cells. Yet both of these cell types show little if any evidence of STC gene expression (Deol *et al.*, 2000; Varghese *et al.*, 1998).

The same discordant patterns of mRNA and protein have been catalogued during bone and testicular development (Fig. 4), whereby STC is made in undifferentiated mesenchyme cells and sequestered by cells destined to differentiate (Stasko and Wagner, 2001b). The pattern is also seen in the postimplantation mouse uterus (Stasko *et al.*, 2001) and may also occur in tumors. For example, in breast carcinomas, there is intense STCir in tumor cells (McCudden *et al.*, 2004), yet our own *in situ* studies of breast and other tumor types suggest that STC mRNA is primarily localized to the vasculature (Gerritsen and Peale, unpublished observations; Gerritsen *et al.*, 2002; Kahn *et al.*, 2000) (Fig. 5). This, however, may not be the case in all tumors; for example, STC mRNA has been localized to breast cancer cells in some mammary tumors (Bouras *et al.*, 2002).

The discordant patterns of mRNA and protein distribution in kidney led to the formulation of a sequestering hypothesis, whereby STC appeared to be synthesized and released by one cell type and heavily sequestered by its target cells (Wong *et al.*, 1998). The hypothesis was further reinforced by the findings obtained in the ovary (Deol *et al.*, 2000) and in the developing mouse embryo (Stasko and Wagner, 2001b), and it was assumed that the process was in some way receptor-mediated. The more interesting question posed by the hypothesis, however, related to the underlying purpose for sequestering, because the degree to which STC was accumulating in target cells suggested that it did more than simply activate signaling cascades.

A. MITOCHONDRIAL TARGETING OF STC50

To address the sequestering hypothesis, we used electron microscope immunocytochemistry to localize STC at the ultrastructural level within the mouse nephron, the tissue in which sequestering had first been observed. Surprisingly, we found that the majority of colloidal gold particles were preferentially sited over mitochondria. We then conducted cell fractionations on the kidney, spleen, lung, and liver to localize STC more precisely at the subcellular level. Surprisingly, only very low levels of STC were found

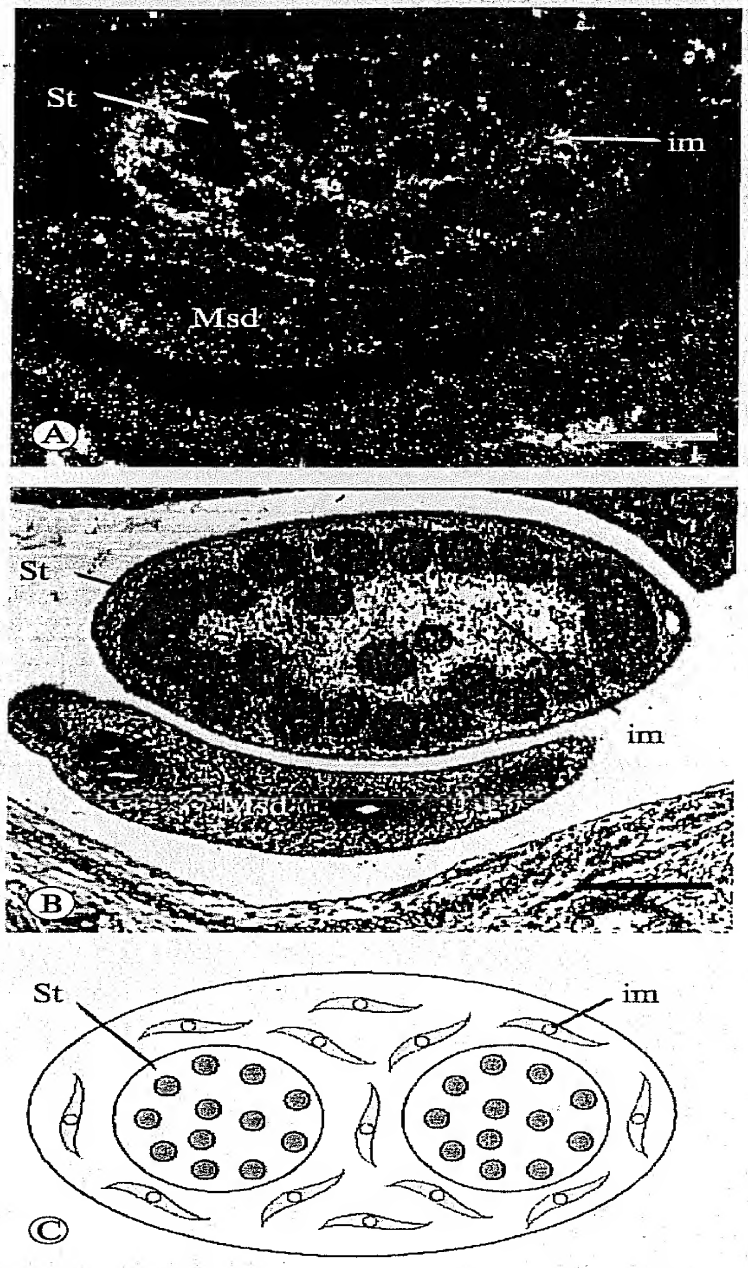


FIGURE 4. STC sequestering in the e14.5 developing mouse testes. (A) A testis following isotopic *in situ* hybridization with a mouse STC cDNA antisense riboprobe. Hybridization is seen almost exclusively over interstitial mesenchyme (im) cells, the future Leydig cell compartment. No signal is evident over the developing seminiferous tubules (St). (B) Section adjacent to A following immunocytochemical localization of STC protein. Although some STC is evident in interstitial mesenchyme cells (im), the vast majority is found in seminiferous tubules (St) that show no evidence of gene expression. (C) Summary of the findings in A and B. The blue interstitial mesenchyme cells (im) are the sites of STC production and contain both transcript and protein. The pink cells, on the other hand, are immature gonocytes in the seminiferous tubules (St). These cells sequester STC ligand to such an extent (presumably via a receptor-mediated mechanism) that they resemble sites of STC production, in spite of the fact that they contain little or no STC mRNA. Ligand sequestering, such as that shown here, is seen in most, if not all, tissues that possess STC50 or big STC signaling pathways. The data are adapted from Stasko and Wagner (2001b). The calibration bars in panels A and B equal 200 μm. (See Color Insert.)

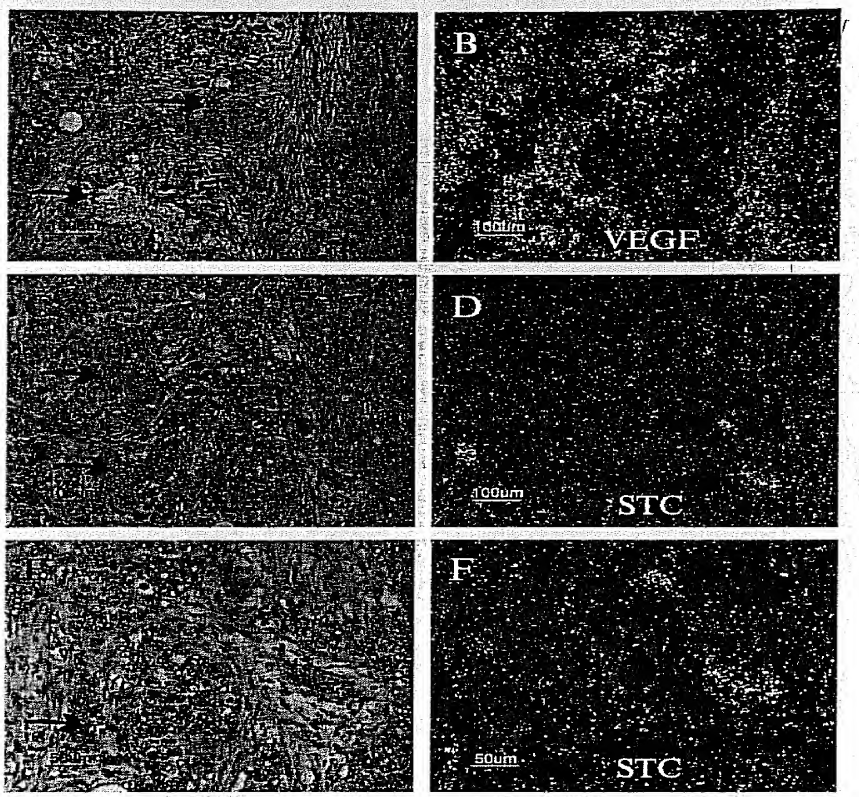


FIGURE 5. STC mRNA is localized to the vasculature of human mammary carcinoma. (A) Bright-field hybridization (B) Dark-field hybridization. Both A and B show *in situ* hybridization studies of VEGF. (C) and (E) Bright-field localization. (D) and (F) Dark-field localization. Panels C, D, E, and F show STC localization in a ductal mammary carcinoma. Note the intense but widespread expression of VEGF mRNA throughout the sample, in contrast to the focal localized expression of STC in a subset of blood vessels adjacent to the tumor cells. Panels E and F are higher power views of a portion of the field shown in C and D. Solid arrows indicate STC expression; double-headed arrows indicate tumor cells. (See Color Insert.)

in the cytosolic fraction, and by far the highest concentrations were found in mitochondria, with at least half in the inner matrix. Western blot analysis revealed that the mitochondria-derived hormone was the same size as rhSTC (i.e., 50 kDa or STC50). Receptor binding studies then delineated the mechanism of entry by revealing there were large numbers of saturable, high-affinity receptors on the plasma membranes as well as on both the outer and inner mitochondrial membranes. That the majority of these receptors were on the inner membrane indicates an inward trafficking pathway for the ligand (Fig. 6). This was subsequently confirmed in trafficking studies on intact, whole mitochondria (McCudden *et al.*, 2002).

Sufficient information is now available to enable at least some speculation about the underlying purpose for mitochondrial targeting. In functional studies on submitochondrial particles, resealed in an inside–out configuration to expose the respiratory chain complex, studies have shown that rhSTC50 has concentration-dependent stimulatory effects on electron

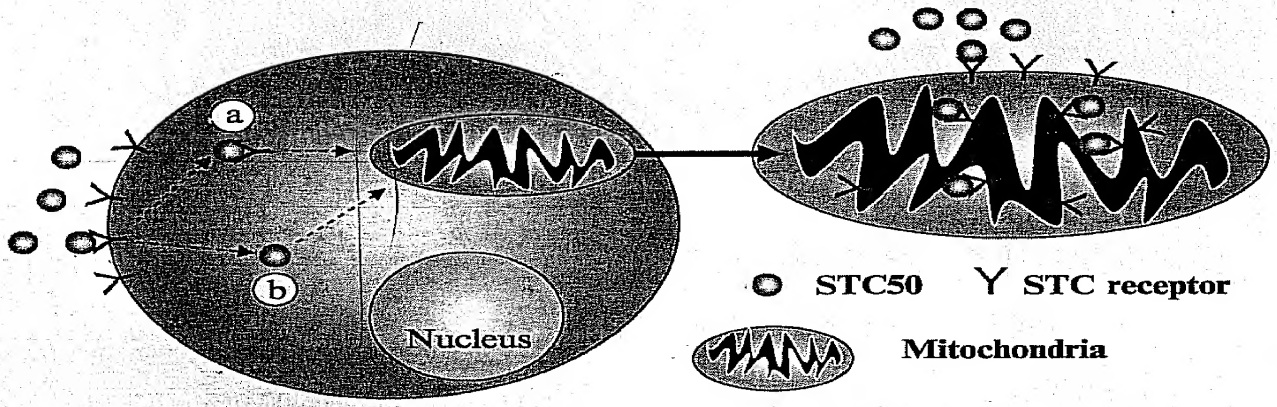


FIGURE 6. Proposed model of mitochondrial targeting by STC50 in nephron epithelial cells. Collecting duct cell-derived STC50 binds to and is internalized by membrane-associated receptors on more upstream nephron segments (paracrine loop) and on collecting duct cells (autocrine loop). Two different pathways are then possible. The ligand may shuttle to the mitochondria together with its receptor (a), or the ligand can shuttle on its own (b). Receptors on both the outer and inner mitochondrial membranes then presumably allow for translocation of STC50 to the inner matrix, where STC50 stimulates electron transport. The precise details relating to ligand internalization and mitochondrial sequestration have not been worked out.

transport chain activity. It seems unlikely, however, that the effect is intended to boost ATP production. Transgenic mouse studies support this supposition (Section III.C). Mice that overexpress the STC gene not only suffer from dwarfism, but they also consume significantly more food and oxygen than their wild-type counterparts, indicative of some type of energy wasting phenotype (Filvaroff *et al.*, 2002; Varghese *et al.*, 2002). Therefore, if STC is not increasing electron transport to promote ATP synthesis, then what else might it be doing? The answer may lie once more in the transgenic phenotype. An intriguing and unexplained aspect of the transgenic phenotype is overt mitochondrial hypertrophy in tissues targeted by the transgene (skeletal muscle in this case). Mitochondrial hypertrophy can be caused by enhanced calcium transport and osmotic swelling due to opening of the mitochondrial permeability transition pore (Abe *et al.*, 2004). Because mitochondria are known repositories of both calcium and phosphate, the possibility that STC50 plays a regulatory role in the import of calcium and/or phosphate cannot be ignored, especially in view of its well-known effects on the transport of both (Section III.B). Therefore, rather than being coupled to ATP synthesis, STC50 in muscle tissue could very well be driving a mitochondrial transporter.

Assuming that STC50 increases electron transport in all receptor-bearing cells and their mitochondria, the resulting effects may not necessarily be the same in all cell types. For instance, whereas calcium fluxes are of obvious importance to muscle cells, they do not necessarily have the same significance to hepatocytes. Likewise, STC50 targets several different nephron segments in the kidney, each of which has differing transport properties.

What the cells in all targeted segments do have in common, however, are their high energy requirements and large numbers of mitochondria to meet these needs. Thick ascending limb and distal convoluted tubule cells have the highest numbers of mitochondria in the nephron (Kriz and Kaissling, 1992) and, coincidently, the greatest number of STC receptors. Loop of Henle cells, on the other hand, have only modest energy demands, contain few mitochondria, and sequester little if any STC50 (Haddad *et al.*, 1996; McCudden *et al.*, 2002; Wong *et al.*, 1998). Indeed, in most cases, the cells targeted by STC50 have reasonably high energy demands. In this regard, at least one laboratory has provided compelling evidence to suggest that STC50 counteracts the apoptotic tendencies of neuronal cell lines following their exposure to hypoxic stress (Zhang *et al.*, 2000). Therefore, we cannot rule out the possibility that the underlying purpose for mitochondrial targeting is to mitigate against the apoptotic pathway.

Whatever proves to be the case, at the very least it can be said that STC50 has joined an exclusive list of regulatory factors, including nitric oxide (Cadenas *et al.*, 2001), melatonin (Reyes-Toso *et al.*, 2003), and thyroid hormone (Wrutniak-Cabello *et al.*, 2001) that have direct effects on electron transport. The only other polypeptide hormone known to be targeted to mitochondria is TGF-β, coincidently another homodimer (Wrutniak-Cabello *et al.*, 2001). None of these ligands, however, has been described as accumulating within the mitochondria to the extent that STC50 does, and they are not accompanied by their receptors. The challenge now is to develop additional experimental paradigms for exploring the underlying purpose behind mitochondrial targeting of this ligand.

B. BIG STC AND ALTERNATIVE FORMS OF SUBCELLULAR TARGETING

The identification of mitochondrial targeting in tissues, such as liver and kidney, has naturally prompted a similar analysis in the ovary, which has by far the highest levels of STC gene activity in mammals. Here, one would expect that the levels of mitochondrial targeting would be among the highest, given the integral role that they play in the steroidogenic pathway. In the final analysis, however, the truth turned out to be quite different. First of all, western blot analysis revealed that ovarian STC was a structurally unique form of the hormone. Instead of the 50-kDa homodimer found in most tissues, ovarian STC consisted of three higher molecular proteins of 84, 112, and 135 kDa. Second, rather than being concentrated in the mitochondrial fraction, all three proteins were preferentially associated with the cholesterol lipid droplets, where they proved to have regulatory effects on the steroidogenic pathway (Paciga *et al.*, 2003).

For the time being, these three STC variants have been collectively dubbed as "big STC" to distinguish them from the 50-kDa form of the hormone, which is now being referred to as STC50. We have very little knowledge of the structure of the big STC variants and many suppositions. None of the variants have been purified and sequenced, and what is known is based primarily on Western blot analysis of ovarian cell extracts. For instance, like STC50, it appears that the variants have a quaternary structure that is stabilized by disulfide linkages. Chemical reduction of ovarian big STC with agents that cleave disulfide linkages collapses all three proteins into one 45-kDa band (Paciga *et al.*, 2003). This is in contrast to STC50 that collapses into its two 25-kDa subunits. This suggests that each of the variants could be progressively larger multimers of the larger 45-kDa monomer (dimers, trimers, tetramers), joined together in a manner that is, in part, disulfide-linked (Fig. 7). Examples of multimers such as these can be found in the case of sonic hedgehog, where the soluble form of the ligand is a complex of six identical subunits, tethered together by their individual cholesterol moieties (Zeng *et al.*, 2001). STC has not been assayed for lipid content. However, a β-myristylation consensus sequence exists near the N terminus (GAFACL), which presumably could serve a similar purpose. Alternatively, the big STC variants could be heteromeric complexes of the 45-kDa subunit and other proteins.

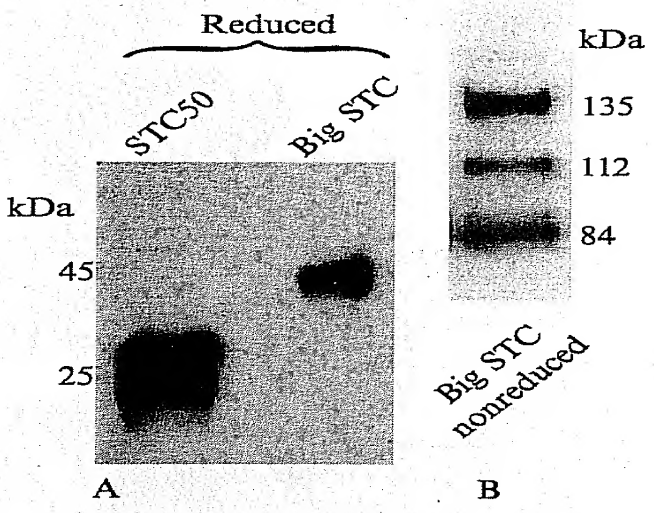


FIGURE 7. Western blot analysis of STC50 and ovarian big STC. (A) The relative mobilities of both proteins on SDS PAGE after chemical reduction with β-mercaptoethanol. The STC50 monomer migrates as a 25-kDa protein versus as a 45-kDa protein for big STC. (B) The migration of big STC in its native state as three higher molecular weight species of 84, 112, and 135 kDa. STC50 (not shown) migrates as one 50-kDa band in the native state. Adapted from Paciga *et al.* (2002).

The nature of the big STC subunit, in particular why it is so much larger, is perhaps the most intriguing question of all. Unlike STC50, big STC has no affinity for the plant lectin, concanavalin A, nor does its electrophoretic mobility seem to be affected by N-glycanase treatment (Paciga *et al.*, 2002), suggesting it is either differentially glycosylated or perhaps not glycosylated at all. What it does share with STC50 is its common cross-reactivity to antibodies generated against rhSTC50 (Niu *et al.*, 2000). Therefore at the very least, they share common antigenic determinants and an unspecified length of core sequence.

C. BIG STC AND ITS INHIBITORY EFFECTS ON OVARIAN LUTEAL CELLS

Within the ovaries, big STC is synthesized in the androgen-producing theca cells and interstitial cells (TICs). From here, it appears to be targeted to developing oocytes and the progesterone-producing luteal cells of the nearby corpus luteum. This is evident by the fact that the majority of the STC receptors are on oocytes and luteal cells, and both cell types contain extraordinarily high levels of STC immunoreactivity. Oocytes have not been examined in any more detail, but in luteal cells, the majority of STC receptors reside on the outer plasma membranes and the hemimembranes surrounding the cholesterol lipid storage droplets (CLDs) that, coincidently, contain high levels of the ligand.

One of the roles of big STC in its targeting of luteal cells is to reduce the production and release of progesterone, which it appears to do by targeting the CLDs (Fig. 8). When added to luteal cells in culture, big STC has dose-dependent inhibitory effects on basal and protein kinase A (PKA)-stimulated progesterone release. Furthermore, the distribution of receptors and their affinities argues in favor of a pathway where the ligand binds first to a plasma membrane-sited receptor, after which it is internalized. The ligand then translocates to the CLDs where there are also large numbers of STC receptors (Fig. 8). It is not known if ligand and receptor traffic together to the CLD via an endosome-type vesicle or if the ligand arrives there on its own and binds to a second receptor subtype. The latter is a distinct possibility because, unlike STC50, whose membrane and mitochondrial receptors are of similar affinity, those on the lipid droplets have a tenfold higher affinity for the ligand ($K_D = 0.1$ nM versus 1 nM for plasma membrane receptors). At present, there is no structural information on STC receptors in any species, and so the physical basis for such marked differences in affinity defy explanation. Functionally, however, the differences in affinity could be essential for inward trafficking of the ligand in the sense that the membrane receptor may function as a low-affinity trap that then passes on captured ligand to the higher affinity CLD receptors.

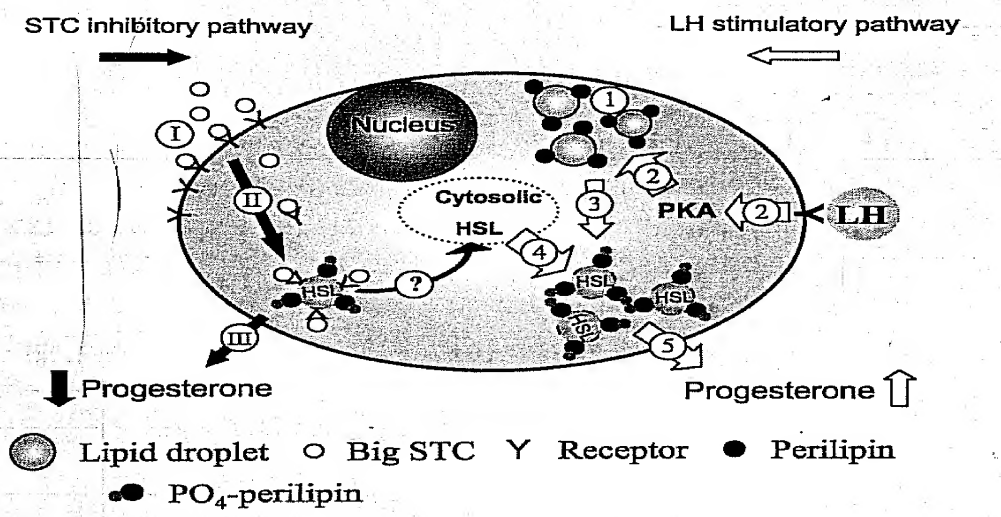


FIGURE 8. Proposed model of big STC subcellular targeting in ovarian luteal cells. In the luteinizing hormone (LH) stimulatory pathway, perilipins coating the cholesterol lipid droplets (CLDs) normally block cholesterol release (1). The binding of LH to its receptor activates the protein kinase A (PKA) pathway (2), and the perilipins are phosphorylated as a result (3), thereby reducing their barrier action. Cytosolic hormone-sensitive lipase (HSL) is also phosphorylated and then translocates to the CLDs to initiate lipolysis (4). Progesterone production and release from stored cholesterol is thereby initiated (5). In the STC inhibitory pathway, big STC of theca cell origin binds to its receptor on the luteal cell membrane (I) and then translocates to the CLD (II). Here, big STC somehow shuts down progesterone production and release (III). In the illustration STC is shown translocating to the CLD with its receptor. However, given that membrane receptors (K_D = 1.0 nM) and CLD receptors (K_D = 0.1 nM) appear to have substantially different ligand affinities, big STC may in fact translocate on its own and bind to a second receptor subtype on the CLD hemimembrane. Following the translocation of big STC and inhibition of progesterone release, the illustration leaves open the option of HSL either remaining with the CLD or returning to the cytosolic pool (?). Precisely how big STC inhibits progesterone release and what its possible interactions are with both the perilipins and HSL are questions that have not been addressed.

The presence of both ligand and receptor on the CLD hemimembrane places STC on a growing, albeit short, list of proteins with regulatory effects on the lipolytic pathway. Notable among these are the perilipins, a group of relatively small proteins that by virtue of their presence on the hemimembrane, block hormone-sensitive lipase (HSL) from accessing stored lipid and releasing cholesterol (Holm, 2003). Access is gained by ligand-induced activation of the protein kinase A (PKA) pathway, whereupon the perilipins and HSL are both phosphorylated, and HSL translocates from the cytosol to the CLD to initiate lipolysis. The mechanism is still poorly understood in terms of precisely how the perilipins block HSL and how variations of this pathway operate in adipocytes (Londos *et al.*, 1999). Ligands such as insulin block the pathway in adipocytes in part by inhibiting PKA and by increasing phosphodiesterase activity within the cells (Shakur *et al.*, 2001). In luteal cells, progesterone synthesis and secretion are inhibited by big STC via mechanisms that are only starting to be explored. Because big STC inhibits

both basal and PKA-activated progesterone release—conditions in which the perilipins are active and inactive, respectively (Londos *et al.*, 1999)—its mechanism of action may preclude involvement with the perilipins. Given that big STC and its receptor establish such a physical presence on the CLD membranes, the mechanism could also entail steric hindrance of HSL.

D. BIG STC SIGNALING DURING PREGNANCY AND LACTATION

Big STC is the first hormone–receptor complex to be identified that targets the CLD, and yet it appears that its actions are not confined to the ovaries or, for that matter, to cholesterol lipid droplets. One of the more intriguing facets of STC biology is the dramatic up-regulation in ovarian hormone production in the mouse during pregnancy and lactation and the appearance of big STC in the serum. The role of the hormone during pregnancy has not been determined. A role in lactation, however, is certainly implied by the fact that enforced weaning reduces ovarian STC expression along with plasma hormone levels down to those of nonpregnancy (Deol *et al.*, 2000). The obvious connection between suckling and ovarian STC production has led us to conclude that ovarian big STC might have a role in mammary gland function, perhaps to aid in milk production.

In the course of pursuing this idea, what has emerged is that mammary gland STC receptors are dramatically up-regulated during pregnancy and even more so during lactation. In contrast to luteal cells, the sites of receptor up-regulation in this case are the plasma membranes and nuclei of the milk-producing alveolar cells (Hasilo *et al.*, 2003). In addition, there appears to be the same type of ligand sequestration by alveolar cell nuclei to the extent that nuclear STC is readily detectable by immunocytochemistry and even quantifiable by immunoassay. Furthermore, because there is greater binding activity on nuclei than on alveolar cell membranes, the receptors appear to be trafficking inward accompanied by the ligand. Western blot analysis has revealed that the sequestered ligand is a big STC variant. Hence, it appears that the ovarian hormone is capable of targeting different subcellular compartments depending on the means of delivery: paracrine or endocrine. The function of big STC in alveolar cells is currently the subject of active study.

V. FUTURE DIRECTIONS

The STC gene is already proving to be highly versatile in terms of the variety of ligands it generates and the scope of their actions on different organelles. Moreover, what has been discovered to date has raised far more questions than have been answered. Preeminent among these questions is the underlying purpose for ligand sequestration. The fact that STC accumulates

at its sites of action to such a great extent suggests that it is doing more than simply activating a signaling cascade. The hormone could be serving as a molecular platform for specific cellular events that, for reasons unknown, require it to be present in large amounts. Alternatively, STC may participate as a substrate in these cellular events. Consideration must also be given to the possibility that biological activity resides in the STC receptor and that the ligand functions simply as a means of directing the receptor to the correct target organelle. This may be relevant in the case of ovarian big STC where the same ligand seems to be targeted to different organelles depending on the means of delivery. This could be indicative of structural differences in the receptors in the different target cell types as well. The sequestering seen in mammalian cells also requires a reappraisal of STC in fishes and, for that matter, invertebrates where STC sequestering is only now being considered. From a purely evolutionary perspective, it is important to know if STC sequestering first evolved in mammals or is in fact widespread throughout the animal kingdom. We suspect the latter will prove to be the case.

Because of our lack of knowledge on receptor structure, one topic that has not been raised in this review concerns the intracellular signaling cascades that might be impacted in response to STC binding to its receptor. The only available information on this subject comes from studies in the fish kidney. These studies have shown that the stimulatory effects of STC on proximal tubular phosphate transport *in vitro* are accompanied by a doubling in cAMP output by the cells. Moreover, the effects of STC are mimicked with the PKA agonist, forskolin, and blocked by the PKA inhibitor, H-89 (Lu *et al.*, 1994). Therefore, in fish kidneys at least, STC seems to exert its effects via the cAMP/PKA pathway. Whether STC activates the same pathway in mammalian kidney has, to our knowledge, not been tested. It is interesting to note, however, that in its inhibitory effects on luteal cells, big STC was capable of reversing PKA-stimulated progesterone release (Paciga *et al.*, 2003), implying that, at the very least, the hormone can impact this pathway. Whether the effects are direct or mediated through an upregulation in phosphodiesterase activity, as in the case of insulin (Londos *et al.*, 1999), is an important question that should now be investigated. Nonetheless, given the data we have at hand, limited though they may be, it appears more than likely that STC will ultimately prove to have regulatory effects on at least some of the known intracellular signaling pathways. What bearing, if any, this might have on STC sequestering remains to be seen.

With respect to the issue of target cell sequestering, it now seems clear that this represents a fundamentally new way of talking between cells. Moreover, it is highly unlikely that STC is the only vertebrate ligand sequestered in this manner. In an article published in the 1980s, investigators working on rat ovary described how the cytoplasm of both oocytes and luteal cells contained sufficiently high enough levels of prolactin and its receptor

that both were readily detectable by immunocytochemistry (Dunaif *et al.*, 1982). Coincidently, these are the same cell types that sequester such high levels of STC and its receptor. To the best of our knowledge, however, these observations stand alone and have not been pursued further. But if one were to fractionate luteal cells, it would not be at all surprising if PRL and its receptor were ultimately found to be heavily concentrated in a discrete subcellular compartment, such as the cholesterol lipid droplet fraction. This may ultimately prove to be the case for other ligands and receptors.

Although direct studies on the regulation of STC gene expression *in vivo* and *in vitro* have been limited, there is a growing body of evidence that STC mRNA and protein levels are increased following the exposure of cells, tissues, and/or animals to stress. Studies using various cultured cell models have shown that STC gene expression is up-regulated by serum (Naranatt *et al.*, 2004); growth factors such as VEGF, bFGF, and HGF (Gerritsen *et al.*, 2003a,b; Liu *et al.*, 2003; Wary *et al.*, 2003; Yang *et al.*, 2002; Zlot *et al.*, 2003); cytokines like TNFα and IL1 (Cooper *et al.*, 2001; Hakonarson *et al.*, 2001; Zlot *et al.*, 2003); acidic pH (Sheikh-Hamad *et al.*, 2000); hypertonicity (Sheikh-Hamad *et al.*, 2000); toxic chemicals (Jellinek *et al.*, 2000); and bacterial and viral infections (Geimonen *et al.*, 2002; Wells *et al.*, 2001). *In vivo, STC* gene expression is elevated at sites of inflammation (Kahn *et al.*, 2000), tissue injury (Kanellis *et al.*, 2004; Sheikh-Hamad *et al.*, 2003), angiogenesis (Gerritsen *et al.*, 2002; Kahn *et al.*, 2000), ischemia (Lal *et al.*, 2001; Zhang *et al.*, 2000), and in various forms of malignancy (Chang *et al.*, 2003; Fujiwara *et al.*, 2000; Lal *et al.*, 2001; McCudden *et al.*, 2004; Miura *et al.*, 2000; Wascher *et al.*, 2003; Welcsh *et al.*, 2002). One could readily speculate that the mitochondrial localization of and effects of STC on metabolic rate and metabolism may be contributing in some way to these physiological responses to stress. This is a final area that clearly warrants further investigation.

ACKNOWLEDGMENTS

We gratefully acknowledge the contributions of the following people to the work reviewed herein: (alphabetically listed) Min Bao, Stuart Bunting, Richard Carano, Ping De Niu, Harminder Deol, Gabe DiMattia, Renee Eckert, Joe Ellard, Ellen Filvaroff, Dorothy French, Bree Flowers, Ryan Gillespie, Susan Guillet, Michel Haddad, Craig Hasilo, John Hoeffel, Gladys Ingle, Kathi James, Jean Kahn, Chris McCudden, Henrik Olsen, Mark Paciga, Franklin Peale, Lyn Powell-Braxton, Dennis Radman, Larry Renfro, Jed Ross, Hope Steinmetz, Sasha Stasko, Cherry Tanega, Robin Varghese, Ben Vozzolo, Chris Wong. Suya Yang, Deenaz Zaidi, and Constance Zlot. Financial support from the Natural Sciences and Engineering Council of Canada, The Canadian Institutes of Health Research, and the Kidney Foundation of Canada to Graham F. Wagner is also gratefully appreciated.

REFERENCES

Abe, T., Takagi, N., Nakano, M., Tanonaka, K., and Takeo, S. (2004). The effects of monobromobimane on calcium- and phenylarsineoxide-induced mitochondrial swelling and cytochrome C release in isolated brain mitochondria. *Biol. Pharm. Bull.* **27,** 524–527.

Amemiya, Y., Marra, L., Reyhani, N., and Youson, J. (2002). Stanniocalcin from an ancient teleost: A monomeric form of the hormone and a possible extracorpuscular distribution. *Mol. Endocrinol.* **188,** 141–150.

Bell, S. E., Mavila, A., Salazar, R., Bayless, K. J., Kanagala, S., Maxwell, S. A., and Davis, G. E. (2001). Differential gene expression during capillary morphogenesis in 3-D collagen matrices: Regulated expression of genes involved in basement membrane matrix assembly, cell cycle progression, cellular differentiation, and G-protein signaling. *J. Cell. Sci.* **114,** 2755–2773.

Bouras, T., Southey, M. C., Chang, A. C., Reddel, R. R., Willhite, D., Glynne, R., Henderson, M. A., Armes, J. E., and Venter, D. J. (2002). Stanniocalcin 2 is an estrogen-responsive gene coexpressed with the estrogen receptor in human breast cancer. *Cancer Res.* **62,** 1289–1295.

Cadenas, E., Poderoso, J. J., Antunes, F., and Boveris, A. (2001). Analysis of the pathways of nitric oxide utilization in mitochondria. *Free Radic. Res.* **33,** 747–756.

Chang, A. C., Dunham, M. A., Jeffrey, K. J., and Reddel, R. R. (1996). Molecular cloning and characterization of mouse stanniocalcin cDNA. *Mol. Cell. Endocrinol.* **124,** 185–187.

Chang, A. C., Janosi, J., Hulsbeek, M., de Jong, D., Jeffrey, K. J., Noble, J. R., and Reddel, R. R. (1995). A novel human cDNA highly homologous to the fish hormone stanniocalcin. *Mol. Cell. Endocrinol.* **112,** 241–247.

Chang, A. C., Jellinek, D. A., and Reddel, R. R. (2003). Mammalian stanniocalcins and cancer. *Endocr. Relat. Cancer* **10,** 359–373.

Chang, A. C., and Reddel, R. R. (1998). Identification of a second stanniocalcin cDNA in mouse and human: Stanniocalcin 2. *Mol. Cell. Endocrinol.* **141,** 95–99.

Cooper, P., Potter, S., Mueck, B., Yousefi, S., and Jarai, G. (2001). Identification of genes induced by inflammatory cytokines in airway epithelium. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **280,** L841–L852.

Deol, H. K., Varghese, R., Wagner, G. F., and Dimattia, G. E. (2000). Dynamic regulation of mouse ovarian stanniocalcin expression during gestation and lactation. *Endocrinology* **141,** 3412–3421.

DiMattia, G. E., Varghese, R., and Wagner, G. F. (1998). Molecular cloning and characterization of stanniocalcin-related protein. *Mol. Cell. Endocrinol.* **146,** 137–140.

Dunaif, A. E., Zimmerman, E. A., Friesen, H. G., and Frantz, A. G. (1982). Intracellular localization of prolactin receptor and prolactin in the rat ovary by immunocytochemistry. *Endocrinology* **110,** 1465–1471.

European Bioinformation Institute (2004). Clustal W Program. http://www.ebi.ac.uk/clustalw/.

Fenwick, J., and So, Y. (1974). A perfusion study of the effect of stanniectomy on the net influx of calcium-45 across an isolated eel gill. *J. Exp. Zool.* **188,** 125–131.

Filvaroff, E. H., Guillet, S., Zlot, C., Bao, M., Ingle, G., Steinmetz, H., Hoeffel, J., Bunting, S., Ross, J., Carano, R. A., Powell-Braxton, L., Wagner, G. F., Eckert, R., Gerritsen, M. E., and French, D. M. (2002). Stanniocalcin 1 alters muscle and bone structure and function in transgenic mice. *Endocrinology* **143,** 3681–3690.

Flik, G. (1990). Hypocalcin physiology. *Prog. Clin. Biol. Res.* **342,** 578–585.

Fontaine, M. (1964). Corpuscules de Stannius et regulation ionique (Ca, K, et Na) du milieu interieur de l'anguille. *CR. Acad. Ser. D* **259,** 875–878.

Fujiwara, Y., Sugita, Y., Nakamori, S., Miyamoto, A., Shiozaki, K., Nagano, H., Sakon, M., and Monden, M. (2000). Assessment of stanniocalcin-1 mRNA as a molecular marker for micrometastases of various human cancers. *Int. J. Oncol.* **16,** 799–804.

Geimonen, E., Neff, S., Raymond, T., Kocer, S. S., Gavrilovskaya, I. N., and Mackow, E. R. (2002). Pathogenic and nonpathogenic hantaviruses differentially regulate endothelial cell responses. *Proc. Natl. Acad. Sci. USA* **99,** 13837–13842.

Gerritsen, M. E., Soriano, R., Yang, S., Ingle, G., Zlot, C., Toy, K., Winer, J., Draksharapu, A., Peale, F., Wu, T. D., and Williams, P. M. (2002). *In silico* data filtering to identify new angiogenesis targets from a large *in vitro* gene profiling data set. *Physiol. Genomics* **10,** 13–20.

Gerritsen, M. E., Soriano, R., Yang, S., Zlot, C., Ingle, G., Toy, K., and Williams, P. M. (2003a). Branching out: A molecular fingerprint of endothelial differentiation into tube-like structures generated by Affymetrix oligonucleotide arrays. *Microcirculation* **10,** 63–81.

Gerritsen, M. E., Tomlinson, J. E., Zlot, C., Ziman, M., and Hwang, S. (2003b). Using gene expression profiling to identify the molecular basis of the synergistic actions of hepatocyte growth factor and vascular endothelial growth factor in human endothelial cells. *Br. J. Pharmacol.* **140,** 595–610.

Haddad, M., Roder, S., Olsen, H. S., and Wagner, G. F. (1996). Immunocytochemical localization of stanniocalcin cells in the rat kidney. *Endocrinology* **137,** 2113–2117.

Hakonarson, H., Halapi, E., Whelan, R., Gulcher, J., Stefansson, K., and Grunstein, M. M. (2001). Association between IL-1beta/TNF-alpha-induced glucocorticoid-sensitive changes in multiple gene expression and altered responsiveness in airway smooth muscle. *Am. J. Respir. Cell. Mol. Biol.* **25,** 761–771.

Hasilo, C., McCudden, C., and Wagner, G. F. (2003). Subcellular targeting of the stanniocalcin ligand and receptor to the nucleus of mammary gland alveolar cells during pregnancy and lactation. *Proc. 85th Ann. Meet. Endo. Soc.*

Holm, C. (2003). Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Biochem. Soc. Trans.* **31,** 1120–1124.

Hulova, I., and Kawauchi, H. (1999). Assignment of disulfide linkages in chum salmon stanniocalcin. *Biochem. Biophys. Res. Commun.* **257,** 295–299.

Ishibashi, K., and Imai, M. (2002). Prospect of a stanniocalcin endocrine/paracrine system in mammals. *Am. J. Physiol. Renal Physiol.* **282,** F367–F375.

Ishibashi, K., Miyamoto, K., Taketani, Y., Morita, K., Takeda, E., Sasaki, S., and Imai, M. (1998). Molecular cloning of a second human stanniocalcin homologue (STC2). *Biochem. Biophys. Res. Commun.* **250,** 252–258.

Jellinek, D. A., Chang, A. C., Larsen, M. R., Wang, X., Robinson, P. J., and Reddel, R. R. (2000). Stanniocalcin 1 and 2 are secreted as phosphoproteins from human fibrosarcoma cells. *Biochem. J.* **350 Pt. 2,** 453–461.

Kahn, J., Mehraban, F., Ingle, G., Xin, X., Bryant, J. E., Vehar, G., Schoenfeld, J., Grimaldi, C. J., Peale, F., Draksharapu, A., Lewin, D. A., and Gerritsen, M. E. (2000). Gene expression profiling in an *in vitro* model of angiogenesis. *Am. J. Pathol.* **156,** 1887–1900.

Kaneko, T., Hasegawa, S., and Hirano, T. (1992). Embryonic origin and development of the corpuscles of Stannius in chum salmon (*Oncorhynchus keta*). *Cell. Tissue Res.* **268,** 65–70.

Kanellis, J., Bick, R., Garcia, G., Truong, L., Tsao, C. C., Etemadmoghadam, D., Poindexter, B., Feng, L., Johnson, R. J., and Sheikh-Hamad, D. (2004). Stanniocalcin 1, an inhibitor of macrophage chemotaxis and chemokinesis. *Am. J. Physiol. Renal Physiol.* **286,** F356–F362.

Krishnamurthy, V. (1976). Cytophysiology of corpuscles of Stannius. *Int. Rev. Cytol.* **46,** 177–249.

Kriz, W., and Kaissling, B. (1992). Structural organization of the mammalian kidney. *In* "The kidney: Physiology and pathophysiology" (D. Seldinand and G. Giebisch, Eds.), Raven Press, New York.

Lafeber, F. P., Hanssen, R. G., Choy, Y. M., Flik, G., Herrmann-Erlee, M. P., Pang, P. K., and Bonga, S. E. (1988). Identification of hypocalcin (teleocalcin) isolated from trout Stannius corpuscles. *Gen. Comp. Endocrinol.* **69,** 19–30.

Lal, A., Peters, H., St Croix, B., Haroon, Z. A., Dewhirst, M. W., Strausberg, R. L., Kaanders, J. H., van der Kogel, A. J., and Riggins, G. J. (2001). Transcriptional response to hypoxia in human tumors. *J. Natl. Cancer Inst.* **93,** 1337–1343.

Lenzmeier, B. A., and Freudenreich, C. H. (2003). Trinucleotide repeat instability: A hairpin curve at the crossroads of replication, recombination, and repair. *Cytogenet. Genome Res.* **100,** 7–24.

Liu, D., Jia, H., Holmes, D. I., Stannard, A., and Zachary, I. (2003). Vascular endothelial growth factor-regulated gene expression in endothelial cells: KDR-mediated induction of Egr3 and the related nuclear receptors Nur77, Nurr1, and Nor1. *Arterioscler. Thromb. Vasc. Biol.* **23,** 2002–2007.

Londos, C., Brasaemle, D. L., Schultz, C. J., Adler-Wailes, D. C., Levin, D. M., Kimmel, A. R., and Rondinone, C. M. (1999). On the control of lipolysis in adipocytes. *Ann. NY Acad. Sci.* **892,** 155–168.

Lu, M., Wagner, G. F., and Renfro, J. L. (1994). Stanniocalcin stimulates phosphate reabsorption by flounder renal proximal tubule in primary culture. *Am. J. Physiol.* **267,** R1356–R1362.

Madsen, K. L., Tavernini, M. M., Yachimec, C., Mendrick, D. L., Alfonso, P. J., Buergin, M., Olsen, H. S., Antonaccio, M. J., Thomson, A. B., and Fedorak, R. N. (1998). Stanniocalcin: A novel protein regulating calcium and phosphate transport across mammalian intestine. *Am. J. Physiol.* **274,** G96–G102.

McCudden, C., Tam, W., and Wagner, G. F. (2001a). Ovarian stanniocalcin in trout is differentially glycosylated and preferentially expressed in early stage oocytes. *Biol. Reprod.* **65,** 763–770.

McCudden, C. R., James, K. A., Hasilo, C., and Wagner, G. F. (2002). Characterization of mammalian stanniocalcin receptors. Mitochondrial targeting of ligand and receptor for regulation of cellular metabolism. *J. Biol. Chem.* **277,** 45249–45258.

McCudden, C. R., Kogon, M. R., Dimattia, G. E., and Wagner, G. F. (2001b). Novel expression of the stanniocalcin gene in fish. *J. Endocrinol.* **171,** 33–44.

McCudden, C. R., Majewski, A., Chakrabarti, S., and Wagner, G. F. (2004). Colocalization of stanniocalcin 1 ligand and receptor in human breast carcinomas. *Mol. Cell. Endocrinol.* **213,** 167–172.

Miura, W., Mizunashi, K., Kimura, N., Koide, Y., Noshiro, T., Miura, Y., Furukawa, Y., and Nagura, H. (2000). Expression of stanniocalcin in zona glomerulosa and medulla of normal human adrenal glands and some adrenal tumors and cell lines. *Apmis* **108,** 367–372.

Moore, E. E., Kuestner, R. E., Conklin, D. C., Whitmore, T. E., Downey, W., Buddle, M. M., Adams, R. L., Bell, L. A., Thompson, D. L., Wolf, A., Chen, L., Stamm, M. R., Grant, F. J., Lok, S., Ren, H., and de Jongh, K. S. (1999). Stanniocalcin 2: Characterization of the protein and its localization to human pancreatic alpha cells. *Horm. Metab. Res.* **31,** 406–414.

Murphy, L. (2003). The role of insulin-like growth factors and their binding proteins in glucose homeostasis. *Exp. Diabesity Res.* **4,** 213–224.

Naranatt, P. P., Krishnan, H. H., Svojanovsky, S. R., Bloomer, C., Mathur, S., and Chandran, B. (2004). Host gene induction and transcriptional reprogramming in Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8)-infected endothelial, fibroblast, and B-cells: Insights into modulation events early during infection. *Cancer Res.* **64,** 72–84.

Niu, P., Radman, D., Jaworski, E., Deol, H., Gentz, R., J, S., Olsen, H., and Wagner, G. F. (2000). Development of a human stanniocalcin radioimmunoassay: Serum and tissue hormone levels and pharmacokinetics in the rat. *Mol. Cell. Endocrinol.* **162,** 131–144.

Ogawa, M. (1967). Fine structure of the corpuscles of Stannius and the interrenal tissue in goldfish (*Carrasius auratus*). *Z. Zellerforsch* **81,** 174–189.

Olsen, H. S., Cepeda, M. A., Zhang, Q. Q., Rosen, C. A., and Vozzolo, B. L. (1996). Human stanniocalcin: A possible hormonal regulator of mineral metabolism. *Proc. Natl. Acad. Sci. USA* **93,** 1792–1796.

Paciga, M., McCudden, C. R., Londos, C., Dimattia, G. E., and Wagner, G. F. (2003). Targeting of big stanniocalcin and its receptor to lipid storage droplets of ovarian steroidogenic cells. *J. Biol. Chem.* **278,** 49549–49554.

Paciga, M., Watson, A. J., Dimattia, G. E., and Wagner, G. F. (2002). Ovarian stanniocalcin is structurally unique in mammals, and its production and release are regulated through the luteinizing hormone receptor. *Endocrinology* **143,** 3925–3934.

Radman, D. P., McCudden, C., James, K., Nemeth, E. M., and Wagner, G. F. (2002). Evidence for calcium-sensing receptor-mediated stanniocalcin secretion in fish. *Mol. Cell. Endocrinol.* **186,** 111–119.

Reyes-Toso, C. F., Ricci, C. R., de Mignone, I. R., Reyes, P., Linares, L. M., Albornoz, L. E., Cardinali, D. P., and Zaninovich, A. (2003). *In vitro* effect of melatonin on oxygen consumption in liver mitochondria of rats. *Neuroendocrinol. Lett.* **24,** 341–344.

Shakur, Y., Holst, L. S., Landstrom, T. R., Movsesian, M., Degerman, E., and Manganiello, V. (2001). Regulation and function of the cyclic nucleotide phosphodiesterase (PDE3) gene family. *Prog. Nucleic Acid Res. Mol. Biol.* **66,** 241–277.

Sheikh-Hamad, D., Bick, R., Wu, G. Y., Christensen, B. M., Razeghi, P., Poindexter, B., Taegtmeyer, H., Wamsley, A., Padda, R., Entman, M., Nielsen, S., and Youker, K. (2003). Stanniocalcin 1 is a naturally occurring L-channel inhibitor in cardiomyocytes: Relevance to human heart failure. *Am. J. Physiol. Heart Circ. Physiol.* **285,** H442–H448.

Sheikh-Hamad, D., Rouse, D., and Yang, Y. (2000). Regulation of stanniocalcin in MDCK cells by hypertonicity and extracellular calcium. *Am. J. Physiol. Renal Physiol.* **278,** F417–F424.

Silberbach, M., and Roberts, C. J. (2001). Natriuretic peptide signalling: Molecular and cellular pathways to growth regulation. *Cell. Signal.* **13,** 221–231.

So, Y., and Fenwick, J. (1979). The *in vivo* and *in vitro* effects of Stannius corpuscles extract on the branchial uptake of 45Ca in stanniectomized North American eel (*Anguilla rostrata*). *Gen. Comp. Endocrinol.* **37,** 143–149.

Stannius, H. (1839). Ober nebenniere bei knochenfischen. *Arch. Anat. Physiol.* **6,** 97–101.

Stasko, S. E., Dimattia, G. E., and Wagner, G. F. (2001). Dynamic changes in stanniocalcin gene expression in the mouse uterus during early implantation. *Mol. Cell. Endocrinol.* **174,** 145–149.

Stasko, S. E., and Wagner, G. F. (2001a). Possible roles for stanniocalcin during early skeletal patterning and joint formation in the mouse. *J. Endocrinol.* **171,** 237–248.

Stasko, S. E., and Wagner, G. F. (2001b). Stanniocalcin gene expression during mouse urogenital development: A possible role in mesenchymal-epithelial signalling. *Dev. Dyn.* **220,** 49–59.

Sundell, K., Bjornsson, B. T., Itoh, H., and Kawauchi, H. (1992). Chum salmon (*Oncorhynchus keta*) stanniocalcin inhibits *in vitro* intestinal calcium uptake in Atlantic cod (*Gadus morhua*). *J. Comp. Physiol. [B]* **162,** 489–495.

Tanega, C., Radman, D., Flowers, B., Sterba, T. A., and Wagner, G. F. (2004). Evidence for stanniocalcin and a related receptor in annelids. *Peptides* **25,** 1671–1679.

Varghese, R., Gagliardi, A. D., Bialek, P. E., Yee, S. P., Wagner, G. F., and Dimattia, G. E. (2002). Overexpression of human stanniocalcin affects growth and reproduction in transgenic mice. *Endocrinology* **143,** 868–876.

Varghese, R., Wong, C. K., Deol, H., Wagner, G. F., and Dimattia, G. E. (1998). Comparative analysis of mammalian stanniocalcin genes. *Endocrinology* **139,** 4714–4725.

Vincent, S. (1898). The effects of extirpation of the supe-renal bodies of the eel (*Anguilla anguilla*). *Proc. Roy Soc. London* **52,** 354.

Wagner, G. F. (1993). Stanniocalcin: Structure, function, and regulation. *In* "The biochemistry and molecular biology of fishes" (P. Hochachkaand and T. Mommsen, Eds.), Vol. 2. Elsevier Science, Amsterdam.

Wagner, G. F. (1994). The molecular biology of the corpuscles of stannius and regulation of stanniocalcin gene expression. *In* "Fish physiology" (N. Sherwoodand and C. Hew, Eds.), Vol. XIII. Academic Press, New York.

Wagner, G. F., Fargher, R. C., Milliken, C., Mckeown, B. A., and Copp, D. H. (1993). The gill calcium transport cycle in rainbow trout is correlated with plasma levels of bioactive, not immunoreactive, stanniocalcin. *Mol. Cell. Endocrinol.* **93,** 185–191.

Wagner, G. F., Guiraudon, C. C., Milliken, C., and Copp, D. H. (1995). Immunological and biological evidence for a stanniocalcin-like hormone in human kidney. *Proc. Natl. Acad. Sci. USA* **92,** 1871–1875.

Wagner, G. F., Hampong, M., Park, C. M., and Copp, D. H. (1986). Purification, characterization, and bioassay of teleocalcin, a glycoprotein from salmon corpuscles of Stannius. *Gen. Comp. Endocrinol.* **63,** 481–491.

Wagner, G. F., Vozzolo, B. L., Jaworski, E., Haddad, M., Kline, R. L., Olsen, H. S., Rosen, C. A., Davidson, M. B., and Renfro, J. L. (1997). Human stanniocalcin inhibits renal phosphate excretion in the rat. *J. Bone Miner. Res.* **12,** 165–171.

Wary, K. K., Thakker, G. D., Humtsoe, J. O., and Yang, J. (2003). Analysis of VEGF-responsive genes involved in the activation of endothelial cells. *Mol. Cancer.* **2,** 25.

Wascher, R. A., Huynh, K. T., Giuliano, A. E., Hansen, N. M., Singer, F. R., Elashoff, D., and Hoon, D. S. (2003). Stanniocalcin 1: A novel molecular blood and bone marrow marker for human breast cancer. *Clin. Cancer Res.* **9,** 1427–1435.

Welcsh, P. L., Lee, M. K., Gonzalez-Hernandez, R. M., Black, D. J., Mahadevappa, M., Swisher, E. M., Warrington, J. A., and King, M. C. (2002). BRCA1 transcriptionally regulates genes involved in breast tumorigenesis. *Proc. Natl. Acad. Sci. USA* **99,** 7560–7565.

Wells, D. B., Tighe, P. J., Wooldridge, K. G., Robinson, K., and Ala' Aldeen, D. A. (2001). Differential gene expression during meningeal–meningococcal interaction: Evidence for self-defense and early release of cytokines and chemokines. *Infect. Immun.* **69,** 2718–2722.

Wendelaar Bonga, S. E., and Pang, P. (1986). Stannius corpuscles. *In* "Vertebrate endocrinology, fundamentals, and biomedical implications" (P. Pangand and S. E. Wendelaar Bonga, Eds.), Vol. I. Academic Press, New York.

Wendelaar Bonga, S. E., and Pang, P. K. (1991). Control of calcium-regulating hormones in the vertebrates: Parathyroid hormone, calcitonin, prolactin, and stanniocalcin. *Int. Rev. Cytol.* **128,** 139–213.

Wong, C. K., Ho, M. A., and Wagner, G. F. (1998). The colocalization of stanniocalcin protein, mRNA, and kidney cell markers in the rat kidney. *J. Endocrinol.* **158,** 183–189.

Wong, C. K., Yeung, H. Y., Mak, N. K., Dimattia, G. E., Chan, D. K., and Wagner, G. F. (2002). Effects of dibutyryl cAMP on stanniocalcin and stanniocalcin-related protein mRNA expression in neuroblastoma cells. *J. Endocrinol.* **173,** 199–209.

Wrutniak-Cabello, C., Casas, F., and Cabello, G. (2001). Thyroid hormone action in mitochondria. *J. Mol. Endocrinol.* **26,** 67–77.

Yang, S., Toy, K., Ingle, G., Zlot, C., Williams, P. M., Fuh, G., Li, B., de Vos, A., and Gerritsen, M. E. (2002). Vascular endothelial growth factor-induced genes in human umbilical vein endothelial cells: Relative roles of KDR and Flt-1 receptors. *Arterioscler. Thromb. Vasc. Biol.* **22,** 1797–1803.

Yeung, H. Y., Chan, D. K., Mak, N. K., Wagner, G. F., and Wong, C. K. (2003). Identification of signal transduction pathways that modulate dibutyryl cyclic adenosine monophosphate activation of stanniocalcin gene expression in neuroblastoma cells. *Endocrinology* **144,** 4446–4452.

Yoshiko, Y., Candeliere, G., Maeda, N., and Aubin, J. (2002). A functional feedback loop comprising stanniocalcin 1, the type III NaPi transporter (Pit1), and Pi transport regulates bone matrix mineralization *in vitro* and *in vivo*. *J. Bone Miner. Res.* **17,** S180.

Yoshiko, Y., Maeda, N., and Aubin, J. E. (2003). Stanniocalcin 1 stimulates osteoblast differentiation in rat calvaria cell cultures. *Endocrinology* **144,** 4134–4143.

Zeng, X., Goetz, J. A., Suber, L. M., Scott, W. J., Jr., Schreiner, C. M., and Robbins, D. J. (2001). A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature.* **411,** 716–720.

Zhang, J., Alfonso, P., Thotakura, N. R., Su, J., Buergin, M., Parmelee, D., Collins, A. W., Oelkuct, M., Gaffney, S., Gentz, S., Radman, D. P., Wagner, G. F., and Gentz, R. (1998). Expression, purification, and bioassay of human stanniocalcin from baculovirus-infected insect cells and recombinant CHO cells. *Protein Expr. Purif.* **12,** 390–398.

Zhang, K., Lindsberg, P. J., Tatlisumak, T., Kaste, M., Olsen, H. S., and Andersson, L. C. (2000). Stanniocalcin: A molecular guard of neurons during cerebral ischemia. *Proc. Natl. Acad. Sci. USA* **97,** 3637–3642.

Zlot, C., Ingle, G., Hongo, J., Yang, S., Sheng, Z., Schwall, R., Paoni, N., Wang, F., Peale, F. V., Jr., and Gerritsen, M. E. (2003). Stanniocalcin 1 is an autocrine modulator of endothelial angiogenic responses to hepatocyte growth factor. *J. Biol. Chem.* **278,** 47654–47659.

patients who have a good performance status, one of the FOLFOX regimens combined with bevacizumab is generally chosen first. Patients are treated to maximal response (commonly judged by radiographic and/or CEA [carcinoembryonic antigen] response) and then given a break from therapy.

There are no firm data regarding continued chemotherapy with or without bevacizumab or continuing bevacizumab by itself when patients are stable. When the disease progresses, one can resume bevacizumab-based therapy. Third-line therapy often consists of other agents, such as cetuximab (Erbitux), which inhibits the epidermal growth factor receptor. In patients who have preexisting neuropathy, one may use irinotecan in place of oxaliplatin. In elderly patients who may have an increased risk of thromboembolic disease and decreased performance status, regimens without bevacizumab are reasonable.

References

1. Poon MA, O'Connell MJ, Moertel CG, et al. Biochemical modulation of fluorouracil: evidence of significant improvement of survival and quality of life in patients with advanced colorectal carcinoma. J Clin Oncol 1989;7:1407–1418.
2. Petrelli N, Herrera L, Rustum Y, et al. A prospective randomized trial of 5-fluorouracil versus 5-fluorouracil and high-dose leucovorin versus 5-fluorouracil and methotrexate in previously untreated patients with advanced colorectal carcinoma. J Clin Oncol 1987;5:1559–1565.
3. Saltz LB, Cox JV, Blanke C, et al. Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. N Engl J Med 2000;343:905–914.
4. Delaunoit T, Goldberg RM, Sargent DJ, et al. Mortality associated with daily bolus 5-fluorouracil/leucovorin administered in combination with either irinotecan or oxaliplatin: results from Intergroup Trial N9741. Cancer 2004;101:2170–2176.
5. Rothenberg ML, Meropol NJ, Poplin EA, Van Cutsem E, Wadler S. Mortality associated with irinotecan plus bolus fluorouracil/ leucovorin: summary findings of an independent panel. J Clin Oncol 2001;18:3801–3807.
6. de Gramont A, Figer A, Seymour M, et al. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. J Clin Oncol 2000;18:2938–2947.
7. Tournigand C, Cervantes A, Figer A, et al. OPTIMOX1: a randomized study of FOLFOX4 or FOLFOX7 with oxaliplatin in a stop-and-go fashion in advanced colorectal cancer—a GERCOR study. J Clin Oncol 2006;24:394–400.
8. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004;350:2335–2342.
9. Giantonio BJ, Levy D, O'Dwyer PJ, Meropol NJ, Catalano PJ, Benson AB. Bevacizumab (anti-VEGF) plus IFL (irinotecan, fluorouracil, leucovorin) as front-line therapy for advanced colorectal cancer (advCRC): results from the Eastern Cooperative Oncology Group (ECOG) Study E2200. Proc Am Soc Clin Oncol 2003;22:1024.
10. Kabbinavar F, Hurwitz HI, Fehrenbacher L, et al. Phase II randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol 2003;21:60–65.
11. Kabbinavarr FF, Schultz J, McCleod M, et al. Addition of bevacizumab to bolus fluorouracil and leucovorin in first-line metastatic colorectal cancer: results of a randomized phase II trial. J Clin Oncol 2005;23:3697–3705.
12. Hochster HS, Hart LL, Ramanathan RK, Hainsworth HD, Hedrick EE, Childs BH. Safety and efficacy of oxaliplatin/fluoropyrimidine regimens with or without bevacizumab as first-line treatment of metastatic colorectal cancer (mCRC): final analysis of the TREE study. J Clin Oncol 2006;(18S):3510.
13. Giantonio BJ, Cataona PJ, Meropol NJ, et al. High-dose bevacizumab improves survival when combined with FOLFOX4 in previously treated advanced colorectal cancer: results from the Eastern Cooperative Oncology Group (ECOG) study E3200. J Clin Oncol 2005;23(16S):2.
14. Giantonio BJ, Chen HX, Catalon PJ, et al. Bowel perforation and fistula formation in colorectal cancer patients treated on Eastern Cooperative Oncology Group (ECOG) studies E2200 and E3200. J Clin Oncol 2004;22(14S):3107.

E-mail Dr. Jones at mjones@wvuh-east.org

From the Perspective of a Pioneer in the Field

Angiogenic inhibitors: a fourth modality of anticancer therapy

Judah Folkman, MD | Children's Hospital of Boston and Harvard Medical School, Boston, MA

In February 2004, Dr. Mark McClellan, the US Food and Drug Administration (FDA) Commissioner, said, "Antiangiogenic therapy can now be considered as the fourth modality of cancer treatment." Currently, nine angiogenic inhibitors have been approved by the FDA to treat cancer, as well as neovascular, age-related macular degeneration (Table 1). These inhibitors are similarly approved in more than 30 other countries. At least 50 other drugs with varying degrees of antiangiogenic activity are in phase II or phase III clinical trials to treat solid tumors and leukemia.

Indirect versus direct inhibition of angiogenesis

Angiogenic inhibitors mainly target activated microvascular endothelial cells in a tumor bed, rather than tumor cells.[1] One class of angiogenic inhibitors, exemplified by endostatin[2] and caplostatin,[3] *directly* prevents the proliferation and motility of endothelial cells that are responding to a wide spectrum of different proangiogenic proteins. Another class of angiogenic inhibitors *indirectly* prevents endothelial-cell proliferation and motility by suppressing a tumor's production of angiogenic proteins (eg, erlotinib [Tarceva]), neutralizing one of

these proteins (eg, bevacizumab [Avastin]), or blocking the endothelial cell receptor for a proangiogenic protein (eg, sunitinib [Sutent]).[1] To date, the majority of FDA-approved angiogenic inhibitors or those in phase III trials act indirectly. However, several inhibitors of the direct type are in phase II clinical trials. For most indirect angiogenic inhibitors, the molecular target is narrow, eg, vascular endothelial growth factor (VEGF) or another proangiogenic protein. Thus, there is a need for combinations of indirect angiogenic inhibitors with each other or with chemotherapy.

Many human tumors, such as breast cancer, can produce up to six or more pro-angiogenic proteins.[4] When these tumors are treated by *indirect* antiangiogenic therapy that blocks only one proangiogenic protein (eg, VEGF), another proangiogenic protein may eventually be produced, such as basic fibroblast growth factor (bFGF), allowing the tumor to evade angiogenic blockade.[5] It is not yet clear whether the emerging bFGF is being produced by the same tumor cells that are producing VEGF or whether different tumor cells are responsible. Nevertheless, clinical experience reveals that it may be prudent for oncologists to continue treatment with an angiogenic inhibitor, even in the face of apparent "drug resistance" (Daniel von Hoff, personal communication). Discontinuing antiangiogenic therapy may lead to more rapid tumor growth. Instead, additional angiogenic inhibitors can be added to broaden the antiangiogenic spectrum, a therapeutic approach called "layering on."

Angiogenic inhibition versus chemotherapy

Other major differences between antiangiogenic therapy and cytotoxic chemotherapy deserve emphasis:

- Virtually all clinically detectable tumors are neovascularized. Some physicians have assumed that a poorly vascularized tumor will not respond to antiangiogenic therapy. But poorly vascularized tumors are inhibited by a significantly lower dose of antiangiogenic therapy than is required for a highly vascularized tumor.
- Although the dose-efficacy curve for chemotherapy is generally a linear function, several angiogenic inhibitors follow a biphasic, U-shaped curve of antiangiogenic and antitumor activity. Lower doses can be more effective than higher doses.[1]
- Although chemotherapy is generally less effective on slowly growing tumors, the opposite is often true of antiangiogenic therapy.
- Optimal antitumor efficacy is usually obtained by sustaining elevated blood levels of angiogenic inhibitors, rather than employing maximum tolerated doses followed by drug-free intervals, as is often done with chemotherapy.
- Finally, patients may remain on antiangiogenic therapy for many years, similar to adjuvant therapy of breast cancer with tamoxifen. Some patients with multiple myeloma have had stable disease without evidence of progression for up to 5 years or more on thalidomide (Thalomid).

In the future, antiangiogenic therapy, in combination with other modalities, may contribute to the conversion of cancer to a chronic manageable disease.[6] As specific and sensitive biomarkers in the blood or urine are developed to identify early tumor angiogenesis, it may eventually be possible to detect recurrent cancer years before symptoms appear—and before anatomic location is possible. A long-term goal would then be to treat recurrent cancer guided by a biomarker without the necessity of locating the tumor, the way infection is treated now.

TABLE 1
Angiogenic inhibitors approved for clinical use

Generic name	Date approved	Country	Therapeutic indication(s)
Bortezomib	May 2003	United States	Multiple myeloma
Thalidomide	December 2003	Australia	Multiple myeloma
Bevacizumab	February 2004	United States	Colorectal cancer
Erlotinib	November 2004	United States	Lung cancer
Bevacizumab	December 2004	Switzerland	Colorectal cancer
Pegaptanib	December 2004	United States	Macular degeneration
Bevacizumab	January 2005	European Union	Colorectal cancer
Endostatin	September 2005	China	Lung cancer
Sorafenib	December 2005	United States	Kidney cancer
Lenalidomide	December 2005	United States	Myelodysplastic syndrome
Sunitinib	January 2006	United States	Gastrointestinal stromal tumor, kidney cancer
Thalidomide	May 2006	United States	Multiple myeloma
Lenalidomide	June 2006	United States	Multiple myeloma
Ranibizumab	June 2006	United States	Macular degeneration
Ranibizumab	August 2006	Switzerland	Macular degeneration
Ranibizumab	September 2006	India	Macular degeneration
Bevacizumab	October 2006	United States	Lung cancer
Ranibizumab	November 2006	European Union*	Macular degeneration
Bevacizumab	March 2007	European Union	Breast cancer

* Provisional approval

References

1. Folkman J. Angiogenesis: an organizing principle for drug discovery. Nat Rev Drug Discov 2007;6:273–286.
2. Abdollahi A, Hahnfeldt P, Maercker C, et al. Endostatin's antiangiogenic signaling network. Mol Cell 2004;13:649–663.
3. Satchi-Fainaro R, Mamluk R, Wang L,

et al. Inhibition of vessel permeability by TNP-470 and its polymer conjugate, caplostatin. Cancer Cell 2005;7:251–261.

4. Relf M, LeJeune S, Fox S, et al. Expression of the angiogenic factors vascular endothelial growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. Cancer Res 1997;57:963–969.

5. Casanovas O, Hicklin DJ, Bergers G, Hanahan D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer Cell 2005;8:299–309.

6. Ezzel C. Starving tumors of their life blood. Sci Am 1998;279:33–44.

E-mail Dr. Folkman at judah.folkman@childrens.harvard.edu.

From the Nurses' Station

Adding bevacizumab to our protocols has improved patient outcomes with little added risk

Andrea Hartman RN, OCN, and Tamara M. Ware, MSN, RN, BC, OCN

City Hospital, a member of West Virginia University Hospitals-East, Martinsburg, WV

Bevacizumab (Avastin), a monoclonal antibody, is an antiangiogenic drug that stops malignant tumors from making new blood vessels. Without their blood supply, tumors cannot grow.

Bevacizumab is generally tolerated well by those receiving it as part of their cancer treatment. It is given over 30–90 minutes intravenously, according to patient tolerance to rate. Bevacizumab has been shown to be effective in non-small cell lung, colorectal, kidney, and breast cancers. The drug has been used at our facility for the past 2 years with good patient response and improvement in quality of life.

Bevacizumab has been used primarily in combination with paclitaxel and also with capecitabine (Xeloda) to treat metastatic breast cancer, the most common cancer in women. Colorectal cancer is the third most common cancer among men and women in the United States. The most widespread use of bevacizumab is in metastatic colorectal cancer. With colorectal cancer, bevacizumab is used in combination with FOLFOX4 (oxaliplatin [Eloxatin], 5-fluorouracil [5-FU], and leucovorin [LV]), FOLFOX6, and FOLFIRI (5-FU, LV, and irinotecan [Camptosar]) regimens. Recent studies have shown that bevacizumab is highly effective in metastatic lung cancer also.

Bevacizumab is contraindicated in patients with hemoptysis or previous surgery in the past 28 days; patients planning to become pregnant or who are pregnant or breastfeeding; and those with certain medical conditions, such as chickenpox, shingles, gout, congestive heart failure and other heart disease, kidney stones, and liver disease. There is an increased risk of blood clots in patients receiving bevacizumab. Bevacizumab should be indefinitely stopped if the patient develops gastrointestinal perforation, wound dehiscence, serious bleeding, a severe arterial thromboembolic event, nephrotic syndrome, or hypertensive encephalopathy.

From the nurse's view, bevacizumab has made great strides in the survival, quality of life, and outcomes of patients. Most of our patients receive their cancer treatment in our outpatient unit. Our patients on bevacizumab for colorectal and breast cancers have had minimal to no complications. The most common side effects from bevacizumab are nausea, vomiting, constipation, headache, epistaxis, and fatigue. Most of our patients have had minimal nausea, vomiting, and constipation. An increase in fatigue has been seen in our patients receiving the every-2-week regimen of FOLFOX4 or FOLFOX6. These patients are able to have these treatments as outpatients with an infusion pump for administering 5-FU at home. Bevacizumab monitoring during and after the infusion was implemented to monitor our patients for any delayed reactions. Thus far, we have had no delayed reactions.

Overall, bevacizumab has provided cancer patients another monoclonal antibody that has minimal side effects compared with those of cytotoxic chemotherapy agents. The advances it has made in cancer treatment are very promising.

E-mail Ms. Hartman at andreahartman@cityhospital.org and Ms. Ware at tware@cityhospital.org.

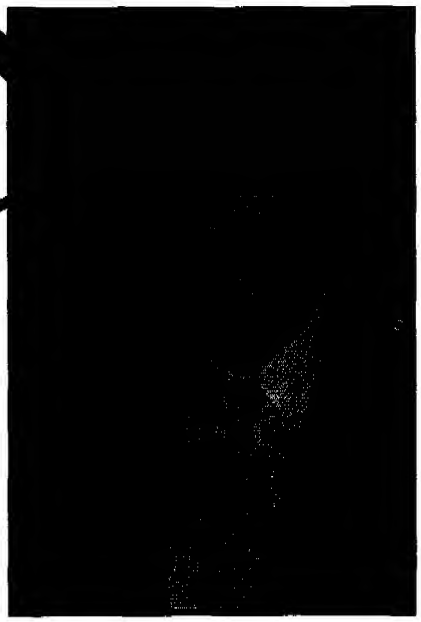
Cancer Research

AN OFFICIAL JOURNAL OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH

CURRENT [illegible] ISSUE

LUDWIG INSTITUTE FOR CANCER RESEARCH

THE FIRST 25 YEARS



INSIDE THIS ISSUE

Call for Abstracts for 1998 AACR Annual Meeting

Abstract Deadline: Oct. 28, 199

October 15, 1997
Volume 57 • Number 20
PP. 4447–4665
ISSN 0008-5472 • CNREA 8

[CANCER RESEARCH 57, 4593–4599, October 15, 1997]

Humanization of an Anti-Vascular Endothelial Growth Factor Monoclonal Antibody for the Therapy of Solid Tumors and Other Disorders

Leonard G. Presta, Helen Chen, Shane J. O'Connor, Vanessa Chisholm, Y. Gloria Meng, Lynne Krummen, Marjorie Winkler, and Napoleone Ferrara[1]

Departments of Immunology, Process Sciences, Molecular Biology, Bioanalytical Technology and Cardiovascular Research, Genentech, Inc., South San Francisco, California 94080

ABSTRACT

Vascular endothelial growth factor (VEGF) is a major mediator of angiogenesis associated with tumors and other pathological conditions, including proliferative diabetic retinopathy and age-related macular degeneration. The murine anti-human VEGF monoclonal antibody (muMAb VEGF) A.4.6.1 has been shown to potently suppress angiogenesis and growth in a variety of human tumor cells lines transplanted in nude mice and also to inhibit neovascularization in a primate model of ischemic retinal disease. In this report, we describe the humanization of muMAb VEGF A.4.6.1. by site-directed mutagenesis of a human framework. Not only the residues involved in the six complementarity-determining regions but also several framework residues were changed from human to murine. Humanized anti-VEGF F(ab) and IgG1 variants bind VEGF with affinity very similar to that of the original murine antibody. Furthermore, recombinant humanized MAb VEGF inhibits VEGF-induced proliferation of endothelial cells *in vitro* and tumor growth *in vivo* with potency and efficacy very similar to those of muMAb VEGF A.4.6.1. Therefore, recombinant humanized MAb VEGF is suitable to test the hypothesis that inhibition of VEGF-induced angiogenesis is a valid strategy for the treatment of solid tumors and other disorders in humans.

INTRODUCTION

It is now well established that angiogenesis is implicated in the pathogenesis of a variety of disorders. These include solid tumors, intraocular neovascular syndromes such as proliferative retinopathies or AMD,[2] rheumatoid arthritis, and psoriasis (1, 2, 3). In the case of solid tumors, the neovascularization allows the tumor cells to acquire a growth advantage and proliferative autonomy compared to the normal cells. Accordingly, a correlation has been observed between density of microvessels in tumor sections and patient survival in breast cancer as well as in several other tumors (4–6).

The search for positive regulators of angiogenesis has yielded several candidates, including acidic fibroblast growth factor (FGF), bFGF, transforming growth factor α, transforming growth factor β, hepatocyte growth factor, tumor necrosis factor-α, angiogenin, interleukin 8, and others (1, 2). However, in spite of extensive research, there is still uncertainty as to their role as endogenous mediators of angiogenesis. The negative regulators thus far identified include thrombospondin (7), the M_r 16,000 NH_2-terminal fragment of prolactin (8), angiostatin (9), and endostatin (10).

Work done over the last several years has established the key role of VEGF in the regulation of normal and abnormal angiogenesis (11). The finding that the loss of even a single VEGF allele results in embryonic lethality points to an irreplaceable role played by this factor in the development and differentiation of the vascular system (11). Also, VEGF has been shown to be a key mediator of neovascularization associated with tumors and intraocular disorders (11). The VEGF mRNA is overexpressed by the majority of human tumors examined (12–16). In addition, the concentration of VEGF in eye fluids is highly correlated to the presence of active proliferation of blood vessels in patients with diabetic and other ischemia-related retinopathies (17). Furthermore, recent studies have demonstrated the localization of VEGF in choroidal neovascular membranes in patients affected by AMD (18).

The muMAb VEGF A.4.6.1 (19) has been used extensively to test the hypothesis that VEGF is a mediator of pathological angiogenesis *in vivo*. This high affinity MAb is able to recognize all VEGF isoforms (19) and has been shown to inhibit potently and reproducibly the growth of a variety of human tumor cell lines in nude mice (11, 20–23). Moreover, intraocular administration of muMAb VEGF A.4.6.1 resulted in virtually complete inhibition of iris neovascularization secondary to retinal ischemia in a primate model (24).

A major limitation in the use of murine antibodies in human therapy is the anti-globulin response (25, 26). Even chimeric molecules, where the variable (V) domains of rodent antibodies are fused to human constant (C) regions, are still capable of eliciting a significant immune response (27). A powerful approach to overcome these limitations in the clinical use of monoclonal antibodies is "humanization" of the murine antibody. This approach was pioneered by Jones *et al.* (28) and Riechman *et al.* (29), who first transplanted the CDRs of a murine antibody into human V domains antibody.

In the present article, we report on the humanization of muMAb VEGF A.4.6.1. Our strategy was to transfer the six CDRs, as defined by Kabat *et al.* (30), from muMAb VEGF A.4.6.1 to a consensus human framework used in previous humanizations (31–33). Seven framework residues in the humanized variable heavy (VH) domain and one framework residue in the humanized variable light (VL) domain were changed from human to murine to achieve binding equivalent to muMAb VEGF A.4.6.1. This humanized MAb is suitable for clinical trials to test the hypothesis that inhibition of VEGF action is an effective strategy for the treatment of cancer and other disorders in humans.

MATERIALS AND METHODS

Cloning of Murine Mab A.4.6.1 and Construction of Mouse-Human Chimeric Fab. Total RNA was isolated from hybridoma cells producing the anti-VEGF MAb A.4.6.1 using RNAsol (Tel-Test) and reverse-transcribed to cDNA using Oligo-dT primer and the SuperScript II system (Life Technologies, Inc., Gaithersburg, MD). Degenerate oligonucleotide primer pools, based of the NH_2-terminal amino acid sequences of the light and heavy chains of the antibody, were synthesized and used as forward primers. Reverse primers were based on framework 4 sequences obtained from murine light chain subgroup κV and heavy chain subgroup II (30). After PCR amplification, DNA fragments were ligated to a TA cloning vector (Invitrogen, San Diego, CA). Eight clones each of the light and

Received 5/27/97; accepted 8/16/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Department of Cardiovascular Research, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080. Phone: (415) 225-2968; Fax: (415) 225-6327; E-mail: Ferrara.Napoleone@gene.com.

[2] The abbreviations used are: AMD, age-related macular degeneration; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; MAb, monoclonal antibody; muMAb, murine MAb; rhuMAb, recombinant humanized MAb; CDR, complementarity-determining region.

heavy chains were sequenced. One clone with a consensus sequence for the light chain VL domain and one with a consensus sequence for the heavy chain VH domain were subcloned, respectively, into the pEMX1 vector containing the human CL and CH1 domains (31), thus generating a mouse-human chimeric F(ab). This chimeric F(ab) consisted of the entire murine A.4.6.1 VH domain fused to a human CH1 domain at amino acid SerH113, and the entire murine A.4.6.1 VL domain fused to a human CL domain at amino acid LysL107. Expression and purification of the chimeric F(ab) were identical to those of the humanized F(ab)s. The chimeric F(ab) was used as the standard in the binding assays.

Computer Graphics Models of Murine and Humanized F(ab)s. Sequences of the VL and VH domains (Fig. 1) were used to construct a computer graphics model of the murine A.4.6.1 VL-VH domains. This model was used to determine which framework residues should be incorporated into the humanized antibody. A model of the humanized F(ab) was also constructed to verify correct selection of murine framework residues. Construction of models was performed as described previously (32, 33).

Construction of Humanized F(ab)s. The plasmid pEMX1 used for mutagenesis and expression of F(ab)s in *Escherichia coli* has been described previously (31). Briefly, the plasmid contains a DNA fragment encoding a consensus human κ subgroup I light chain (VLκI-CL) and a consensus human subgroup III heavy chain (VHIII-CH1) and an alkaline phosphatase promoter. The use of the consensus sequences for VL and VH has been described previously (32).

To construct the first F(ab) variant of humanized A.4.6.1, F(ab)-1, site-directed mutagenesis (34) was performed on a deoxyuridine-containing template of pEMX1. The six CDRs were changed to the murine A.4.6.1 sequence; the residues included in each CDR were from the sequence-based CDR definitions (30). F(ab)-1, therefore, consisted of a complete human framework (VL κ subgroup I and VH subgroup III) with the six complete murine CDR sequences. Plasmids for all other F(ab) variants were constructed from the plasmid template of F(ab)-1. Plasmids were transformed into *E. coli* strain XL-1 Blue (Stratagene, San Diego, CA) for preparation of double- and single-stranded DNA. For each variant, DNA coding for light and heavy chains was completely sequenced using the dideoxynucleotide method (Sequenase; U.S. Biochemical Corp., Cleveland, OH). Plasmids were transformed into *E. coli* strain 16C9, a derivative of MM294, plated onto Luria broth plates containing 50 μg/ml carbenicillin, and a single colony selected for protein expression. The single colony was grown in 5 ml of Luria broth-100 μg/ml carbenicillin for 5–8 h at 37°C. The 5-ml culture was added to 500 ml of AP5–50 μg/ml carbenicillin and allowed to grow for 20 h in a 4-liter baffled shake flask at 30°C. AP5 media consists of 1.5 g of glucose, 11.0 g of Hycase SF, 0.6 g of yeast extract (certified), 0.19 g of $MgSO_4$ (anhydrous), 1.07 g of NH_4Cl, 3.73 g of KCl, 1.2 g of NaCl, 120 ml of 1 M triethanolamine, pH 7.4, to 1 liter of water and then sterile filtered through a 0.1-mm Sealkeen filter. Cells were harvested by centrifugation in a 1-liter centrifuge bottle at 3000 × *g*, and the supernatant was removed. After freezing for 1 h, the pellet was resuspended in 25 ml of cold 10 mM Tris, 1 mM EDTA, and 20% sucrose, pH 8.0. Two hundred fifty ml of 0.1 M benzamidine (Sigma Chemical Co., St. Louis, MO) was added to inhibit proteolysis. After gentle stirring on ice for 3 h, the sample was centrifuged at 40,000 × *g* for 15 min. The supernatant was then applied to a protein G-Sepharose CL-4B (Pharmacia Biotech, Inc., Uppsala, Sweden) column (0.5-ml bed volume) equilibrated with 10 mM Tris-1 mM EDTA, pH 7.5. The column was washed with 10 ml of 10 mM Tris-1 mM EDTA, pH 7.5, and eluted with 3 ml of 0.3 M glycine, pH 3.0, into 1.25 ml of 1 M Tris, pH 8.0. The F(ab) was then buffer exchanged into PBS using a Centricon-30 (Amicon, Beverly, MA) and concentrated to a final volume of 0.5 ml. SDS-PAGE gels of all F(ab)s were run to ascertain purity, and the molecular weight of each variant was verified by electrospray mass spectrometry.

Construction, Expression, and Purification of Chimeric and Humanized IgG Variants. For the generation of human IgG1 variants of chimeric (chIgG1) and humanized (rhuMAb VEGF) A.4.6.1, the appropriate murine or humanized VL and VH (F(ab)-12; Table 1) domains were subcloned into separate, previously described pRK vectors (35). The DNA coding for the entire light and the entire heavy chain of each variant was verified by dideoxynucleotide sequencing.

For transient expression of variants, heavy and light chain plasmids were cotransfected into human 293 cells (36) using a high efficiency procedure (37). Media were changed to serum free and harvested daily for up to 5 days. Antibodies were purified from the pooled supernatants using protein A-Sepharose CL-4B (Pharmacia). The eluted antibody was buffer exchanged into PBS using a Centricon-30 (Amicon), concentrated to 0.5 ml, sterile filtered using a Millex-GV (Millipore, Bedford, MA), and stored at 4°C.

For stable expression of the final humanized IgG1 variant (rhuMAb VEGF), Chinese hamster ovary (CHO) cells were transfected with dicistronic vectors designed to coexpress both heavy and light chains (38). Plasmids were introduced into DP12 cells, a proprietary derivative of the CHO-K1 DUX B11 cell line developed by L. Chasin (Columbia University, New York, NY), via lipofection and selected for growth in glycine/hypoxanthine/thymidine (GHT)-free medium (39). Approximately 20 unamplified clones were randomly chosen and reseeded into 96-well plates. Relative specific productivity of each colony was monitored using an ELISA to quantitate the full-length human IgG accumulated in each well after 3 days and a fluorescent dye, Calcien AM, as a surrogate marker of viable cell number per well. Based on these data, several unamplified clones were chosen for further amplification in the presence of increasing concentrations of methotrexate. Individual clones surviving at 10, 50, and 100 nM methotrexate were chosen and transferred to 96-well plates for productivity screening. One clone, which reproducibly exhibited high specific productivity, was expanded in T-flasks and used to inoculate a spinner

Table 1 *Binding of humanized anti-VEGF F(ab) variants to VEGF*[a]

Variant	Template	Changes[b]	Purpose	EC50 F(ab)-X EC_{50} chimeric F(ab)[c] Mean	SD	N
chim-F(ab)	Chimeric F(ab)		1.0			
F(ab)-1	Human FR		Straight CDR swap	>1350		2
F(ab)-2			Chimera light chain	>145		3
			F(ab)-1 heavy chain			
F(ab)-3			F(ab)-1 light chain	2.6	0.1	2
			Chimera heavy chain			
F(ab)-4	F(ab)-1	ArgH71Leu	CDR-H2 conformation	>295		3
		AspH73Asn	Framework			
F(ab)-5	F(ab)-4	LeuL46Val	VL-VH interface	80.9	6.5	2
F(ab)-6	F(ab)-5	LeuH78Ala	CDR-H1 conformation	36.4	4.2	2
F(ab)-7	F(ab)-5	IleH69Phe	CDR-H2 conformation	45.2	2.3	2
F(ab)-8	F(ab)-5	IleH69Phe	CDR-H2 conformation	9.6	0.9	4
		LeuH78Ala	CDR-H1 conformation			
F(ab)-9	F(ab)-8	GlyH49Ala	CDR-H2 conformation	>150		2
F(ab)-10	F(ab)-8	AsnH76Ser	Framework	6.4	1.2	4
F(ab)-11	F(ab)-10	LysH75Ala	Framework	3.3	0.4	2
F(ab)-12	F(ab)-10	ArgH94Lys	CDR-H3 conformation	1.6	0.6	4

[a] Anti-VEGF F(ab) variants were incubated with biotinylated VEGF and then transferred to ELISA plates coated with KDR-IgG (40).
[b] Murine residues are underlined; residue numbers are according to Kabat *et al.* (30).
[c] Mean and SD are the average of the ratios calculated for each of the independent assays; the EC_{50} for chimeric F(ab) was 0.049 ± 0.013 mg/ml (1.0 nM).

culture. After several passages, the suspension-adapted cells were used to inoculate production cultures in GHT-containing, serum-free media supplemented with various hormones and protein hydrolysates. Harvested cell culture fluid containing rhuMAb VEGF was purified using protein A-Sepharose CL-4B. The purity after this step was ~99%. Subsequent purification to homogeneity was carried out using an ion exchange chromatography step. The endotoxin content of the final purified antibody was <0.10 eu/mg.

F(ab) and IgG Quantitation. For quantitating F(ab) molecules, ELISA plates were coated with 2 μg/ml of goat anti-human IgG Fab (Organon Teknika, Durham, NC) in 50 mM carbonate buffer, pH 9.6, at 4°C overnight and blocked with PBS-0.5% BSA (blocking buffer) at room temperature for 1 h. Standards [0.78–50 ng/ml human F(ab)] were purchased from Chemicon (Temecula, CA). Serial dilutions of samples in PBS-0.5% BSA-0.05% polysorbate 20 (assay buffer) were incubated on the plates for 2 h. Bound F(ab) was detected using horseradish peroxidase-labeled goat anti-human IgG F(ab) (Organon Teknika), followed by 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as the substrate. Plates were washed between steps. Absorbance was read at 450 nm on a V_{max} plate reader (Molecular Devices, Menlo Park, CA). The standard curve was fit using a four-parameter nonlinear regression curve-fitting program developed at Genentech. Data points that fell in the range of the standard curve were used for calculating the F(ab) concentrations of samples.

The concentration of full-length antibody was determined using goat anti-human IgG Fc (Cappel, Westchester, PA) for capture and horseradish peroxidase-labeled goat anti-human Fc (Cappel) for detection. Human IgG1 (Chemicon) was used as standard.

VEGF Binding Assays. For measuring the VEGF binding activity of F(ab)s, ELISA plates were coated with 2 μg/ml rabbit $F(ab')_2$ to human IgG Fc (Jackson ImmunoResearch, West Grove, PA) and blocked with blocking buffer (described above). Diluted conditioned medium containing 3 ng/ml of KDR-IgG (40) in blocking buffer were incubated on the plate for 1 h. Standards [6.9–440 ng/ml chimeric F(ab)] and 2-fold serial dilutions of samples were incubated with 2 nM biotinylated VEGF for 1 h in tubes. The solutions from the tubes were then transferred to the ELISA plates and incubated for 1 h. After washing, biotinylated VEGF bound to KDR was detected using horseradish peroxidase-labeled streptavidin (Zymed, South San Francisco, CA or Sigma) followed by 3,3′,5,5′-tetramethylbenzidine as the substrate. Titration curves were fit with a four-parameter nonlinear regression curve-fitting program (KaleidaGraph; Synergy Software, Reading, PA). Concentrations of F(ab) variants corresponding to the midpoint absorbance of the titration curve of the standard were calculated and then divided by the concentration of the standard corresponding to the midpoint absorbance of the standard titration curve. Assays for full-length IgG were the same as for the F(ab)s except that the assay buffer contained 10% human serum.

BIAcore Biosensor Assays. VEGF binding of the humanized and chimeric F(ab)s were compared using a BIAcore biosensor (41). Concentrations of F(ab)s were determined by quantitative amino acid analysis. VEGF was coupled to a CM-5 biosensor chip through primary amine groups according to manufacturer's instructions (Pharmacia). Off-rate kinetics were measured by saturating the chip with F(ab) [35 μl of 2 μM F(ab) at a flow rate of 20 μl/min] and then switching to buffer (PBS-0.05% polysorbate 20). Data points from 0–4500 s were used for off-rate kinetic analysis. The dissociation rate constant (k_{off}) was obtained from the slope of the plot of $\ln(R0/R)$ *versus* time, where $R0$ is the signal at $t = 0$ and R is the signal at each time point.

On-rate kinetics were measured using 2-fold serial dilutions of F(ab) (0.0625–2 mM). The slope, K_s, was obtained from the plot of $\ln(-dR/dt)$ *versus* time for each F(ab) concentration using the BIAcore kinetics evaluation software as described in the Pharmacia Biosensor manual. R is the signal at time t. Data between 80 and 168, 148, 128, 114, 102, and 92 s were used for 0.0625, 0.125, 0.25, 0.5, 1, and 2 mM F(ab), respectively. The association rate constant (k_{on}) was obtained from the slope of the plot of K_s *versus* F(ab) concentration. At the end of each cycle, bound F(ab) was removed by injecting 5 μl of 50 mM HCl at a flow rate of 20 μl/min to regenerate the chip.

Endothelial Cell Growth Assay. Bovine adrenal cortex-derived capillary endothelial cells were cultured in the presence of low glucose DMEM (Life Technologies, Inc.) supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium), essentially as described previously (42). For mitogenic assays, endothelial cells were seeded at a density of 6×10^3 cells/well in 6-well plates in growth medium. Either muMAb VEGF A.4.6.1 or rhuMAb VEGF was then added at concentrations ranging between 1 and 5000 ng/ml. After 2–3 h, purified *E. coli*-expressed $rhVEGF_{165}$ was added to a final concentration of 3 ng/ml. For specificity control, each antibody was added to endothelial cells at the concentration of 5000 ng/ml, either alone or in the presence of 2 ng/ml bFGF. After 5 or 6 days, cells were dissociated by exposure to trypsin, and duplicate wells were counted in a Coulter counter (Coulter Electronics, Hialeah, FL). The variation from the mean did not exceed 10%. Data were analyzed by a four-parameter curve fitting program (KaleidaGraph).

***In Vivo* Tumor Studies.** Human A673 rhabdomyosarcoma cells (American Type Culture Collection; CRL 1598) were cultured as described previously in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics (20, 22). Female BALB/c nude mice, 6–10 weeks old, were injected s.c. with 2×10^6 tumor cells in the dorsal area in a volume of 200 μl. Animals were then treated with muMAb VEGF A.4.6.1, rhuMAb VEGF, or a control murine MAb directed against the gp120 protein. Both anti-VEGF MAbs were administered at the doses of 0.5 and 5 mg/kg; the control MAb was given at the dose of 5 mg/kg. Each MAb was administered twice weekly i.p. in a volume of 100 μl, starting 24 h after tumor cell inoculation. Each group consisted of 10 mice. Tumor size was determined at weekly intervals. Four weeks after tumor cell inoculation, animals were euthanized, and the tumors were removed and weighed. Statistical analysis was performed by ANOVA.

RESULTS

Humanization. The consensus sequence for the human heavy chain subgroup III and the light chain subgroup κ I were used as the framework for the humanization (Ref. 30; Fig. 1). This framework has been successfully used in the humanization of other murine antibodies (31, 32, 43, 44). All humanized variants were initially made and screened for binding as F(ab)s expressed in *E. coli*. Typical yields from 500-ml shake flasks were 0.1–0.4 mg F(ab).

Two definitions of CDR residues have been proposed. One is based on sequence hypervariability (30) and the other on crystal structures of F(ab)-antigen complexes (45). The sequence-based CDRs are larger than the structure-based CDRs, and the two definitions are in agreement except for CDR-H1; CDR-H1 includes residues H31–H35 according to the sequence-based definition, and residues H26–H32 according to the structure-based definition (light chain residue numbers are prefixed with L; heavy chain residue numbers are prefixed with H). We, therefore, defined CDR-H1 as a combination of the two, *i.e.*, including residues H26–H35. The other CDRs were defined using the sequence-based definition (30).

The chimeric F(ab) was used as the standard in the binding assays. In the initial variant, F(ab)-1, the CDR residues were transferred from the murine antibody to the human framework and, based on the models of the murine and humanized F(ab)s, the residue at position H49 (Ala in humans) was changed to the murine Gly. In addition, F(ab)s that consisted of the chimeric heavy chain/F(ab)-1 light chain [F(ab)-2] and F(ab)-1 heavy chain/chimeric light chain [F(ab)-3] were generated and tested for binding. F(ab)-1 exhibited a binding affinity greater than 1000-fold reduced from the chimeric F(ab) (Table 1). Comparing the binding affinities of F(ab)-2 and F(ab)-3 suggested that framework residues in the F(ab)-1 VH domain needed to be altered to increase binding.

Previous humanizations (31, 32, 43, 44) as well as studies of F(ab)-antigen crystal structures (45, 47) have shown that residues H71 and H73 can have a profound effect on binding, possibly by influencing the conformations of CDR-H1 and CDR-H2. Changing the human residues to their murine counterparts in F(ab)-4 improved binding by 4-fold (Table 1). Inspection of the models of the murine

Variable Heavy

```
A.4.6.1    EIQLVQSGPELKQPGETVRISCKASGYTFTNYGMNWVKQAPGKGLKWMG
           *   *  ** *   *** * *             *       * *
F(ab)-12   EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVG
                                     * ** * *             *
humIII     EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVS
           1       10        20        30        40

A.4.6.1    WINTYTGEPTYAADFKRRFTFSLETSASTAYLQISNLKNDDTATYFCAK
                                    * *      *** ***   * *
F(ab)-12   WINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAK
           * **** ***  *** *  * * *  * *                   *
humIII     VISGDGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR
          50  a      60        70        80  abc       90

A.4.6.1    YPHYYGSSHWYFDVWGAGTTVTVSS
                          * *
F(ab)-12   YPHYYGSSHWYFDVWGQGTLVTVSS
           *           *
humIII     G----------FDYWGQGTLVTVSS
                         110
```

Variable Light

```
A.4.6.1    DIQMTQTTSSLSASLGDRVIISCSASQDISNYLNWYQQKPDGTVKVLIY
                 **      *   * *             ****
F(ab)-12   DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIY
                                  * *     *         *
humKI      DIQMTQSPSSLSASVGDRVTITCRASQSISNYLAWYQQKPGKAPKLLIY
           1       10        20        30        40

A.4.6.1    FTSSLHSGVPSRFSGSGSGTDYSLTISNLEPEDIATYYCQQYSTVPWTF
                                  **    * *   *
F(ab)-12   FTSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTF
           **   *                                    ***
humKI      AASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPWTF
          50        60        70        80        90

A.4.6.1    GGGTKLEIKR
           *   *
F(ab)-12   GQGTKVEIKR

humKI      GQGTKVEIKR
          100
```

Fig. 1. Amino acid sequence of variable heavy and light domains of muMAbVEGF A.4.6.1, humanized F(ab) with optimal VEGF binding [F(ab)-12] and human consensus frameworks (*humIII*, heavy subgroup III; *humκ1*, light κ subgroup I). *Asterisks*, differences between humanized F(ab)-12 and the murine MAb or between F(ab)-12 and the human framework. CDRs are *underlined*.

and humanized F(ab)s suggested that residue L46, buried at the VL-VH interface and interacting with CDR-H3 (Fig. 2), might also play a role either in determining the conformation of CDR-H3 and/or affecting the relationship of the VL and VH domains. When the murine Val was exchanged for the human Leu at L46 [F(ab)-5], the binding affinity increased by almost 4-fold (Table 1). Three other buried framework residues were evaluated based on the molecular models: H49, H69, and H78. Position H69 may affect the conformation of CDR-H2, whereas position H78 may affect the conformation of CDR-H1 (Fig. 2). When each was individually changed from the human to murine counterpart, the binding improved by 2-fold in each case [F(ab)-6 and F(ab)-7; Table 1]. When both were simultaneously changed, the improvement in binding was 8-fold [F(ab)-8; Table 1]. Residue H49 was originally included as the murine Gly; when changed to the human consensus counterpart Ala, the binding was reduced by 15-fold [F(ab)-9; Table 1].

We have found during previous humanizations that residues in a framework loop, FR-3 (30) adjacent to CDR-H1 and CDR-H2, can affect binding (44). In F(ab)-10 and F(ab)-11, two residues in this loop were changed to their murine counterparts: AsnH76 to murine Ser [F(ab)-10] and LysH75 to murine Ala [F(ab)-11]. Both effected a relatively small improvement in binding (Table 1). Finally, at position H94, human and murine sequences most often have an Arg (30). In F(ab)-12, this Arg was replaced by the rare Lys found in the murine antibody (Fig. 1), and this resulted in binding that was less than 2-fold from the chimeric F(ab) (Table 1). F(ab)-12 was also compared to the chimeric F(ab) using the BIAcore system (Pharmacia). Using this technique, the K_d of the humanized F(ab)-12 was 2-fold weaker than that of the chimeric F(ab) due to both a slower k_{on} and faster k_{off} (Table 2).

Full-length MAbs were constructed by fusing the VL and VH domains of the chimeric F(ab) and variant F(ab)-12 to the constant domains of human κ light chain and human IgG1 heavy chain. The full-length 12-IgG1 [F(ab)-12 fused to human IgG1] exhibited binding that was 1.7-fold weaker than the chimeric IgG1 (Table 3). Both 12-IgG1 and the chimeric IgG1 bound slightly less well than the original muMAb VEGF A.4.6.1 (Table 3).

Biological Studies. rhuMAb VEGF and muMAb VEGF A.4.6.1 were compared for their ability to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of $VEGF_{165}$ (3 ng/ml). In several experiments, the two MAbs were found to be essentially equivalent, both in potency and efficacy. The ED_{50}s were, respectively, 50 ± 5 and 48 ± 8 ng/ml (~0.3 nM). In both cases, 90% inhibition was achieved at the concentration of 500 ng/ml (~3 nM). Fig. 3 illustrates a representative experiment. Neither muMAb VEGF A.4.6.1 nor rhuMAb VEGF had any effect on basal or bFGF-stimulated proliferation of capillary endothelial cells (data not shown), confirming that the inhibition is specific for VEGF.

To determine whether similar findings could be obtained also in an *in vivo* system, we compared the two antibodies for their ability to suppress the growth of human A673 rhabdomyosarcoma cells in nude mice. Previous studies have shown that muMAb VEGF A.4.6.1 has a dramatic inhibitory effect in this tumor model (20, 22). As shown in Fig. 4, at both doses tested (0.5 and 5 mg/kg), the two antibodies markedly suppressed tumor growth as assessed by tumor weight measurements 4 weeks after cell inoculation. The decreases in tumor weight compared to the control group were, respectively, 85 and 93% at each dose in the animals treated with muMAb VEGF A.4.6.1 *versus* 90 and 95% in those treated with rhuMAb VEGF. Similar results were obtained with the breast carcinoma cell line MDA-MB 435 (data not shown).

DISCUSSION

The murine MAb A.4.6.1, directed against human VEGF (42), was humanized using the same consensus frameworks for the light and heavy chains used in previous humanizations (31, 32, 43, 44), *i.e.*, VκI and VHIII (30). Simply transferring the CDRs from the murine antibody to the human framework resulted in a F(ab) that exhibited binding to VEGF reduced by over 1000-fold compared to the parent murine antibody. Seven non-CDR, framework residues in the VH domain and one in the VL domain were altered from human to murine to achieve binding equivalent to the parent murine antibody.

In the VH domain, residues at positions H49, H69, H71, and H78 are buried or partially buried and probably effect binding by influencing the conformation of the CDR loops. Residues H73 and H76 should be solvent exposed (Fig. 2) and hence may interact directly with the VEGF; these two residues are in a non-CDR loop adjacent to CDRs H1 and H2 and have been shown to play a role in binding in previous humanizations (31, 32, 44). The requirement for lysine at position H94 was surprising given that this residue is arginine in the human framework (Fig. 1). In some crystal structures of F(ab)s, ArgH94 forms a hydrogen-bonded salt-bridge with

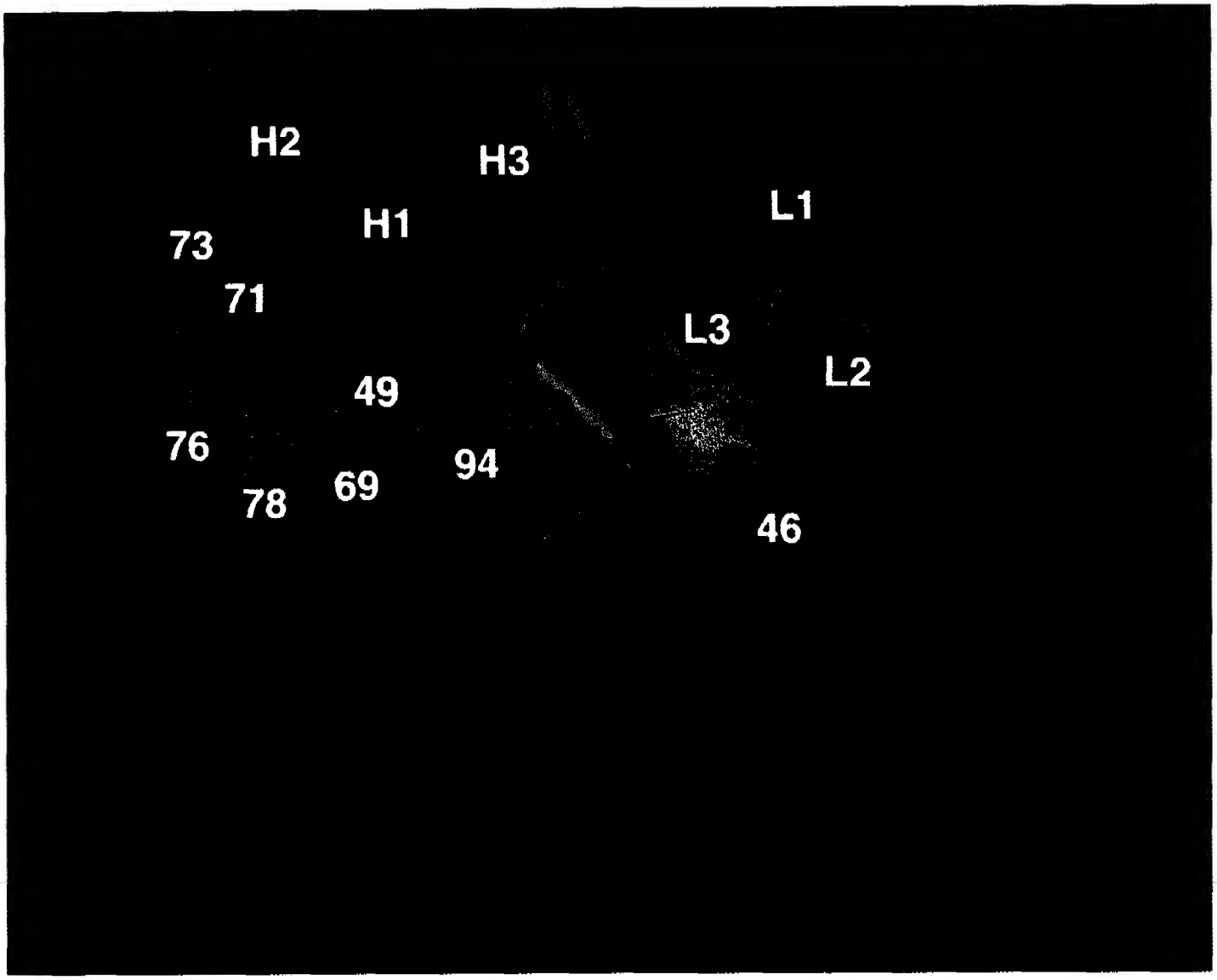


Fig. 2. Ribbon diagram of the model of humanized F(ab)-12 VL and VH domains. VL domain is shown in *brown* with CDRs in *tan*. The side chain of residue L46 is shown in *yellow*. VH domain is shown in *purple* with CDRs in *pink*. Side chains of VH residues changed from human to murine are shown in *yellow*.

Table 2 *Binding of anti-VEGF F(ab) variants to VEGF using the BIAcore system*[a]

Variant	Amount of (Fab) bound (RU)	k_{off} (s^{-1})	k_{on} ($M^{-1}s^{-1}$)	K_d (nM)
chim-F(ab)[b]	4250	5.9×10^{-5}	6.5×10^{4}	0.91
F(ab)-12	3740	6.3×10^{-5}	3.5×10^{4}	1.8

[a] The amount of F(ab) bound, in resonance units (RU), was measured using a BIAcore system when 2 μg F(ab) was injected onto a chip containing 2480 RU of immobilized VEGF. Off-rate kinetics (k_{off}) were measured by saturating the chip with F(ab) and then monitoring dissociation after switching to buffer. On-rate kinetics (k_{on}) were measured using 2-fold serial dilutions of F(ab). K_d, the equilibrium dissociation constant, was calculated as k_{off}/k_{on}.

[b] chim-F(ab) is a chimeric F(ab) with murine VL and VH domains fused to human CL and CH1 heavy domains.

AspH101 (33, 48). Substitution of lysine for arginine might conceivably alter this salt-bridge and perturb the conformation of CDR-H3.

In the VL domain, only one framework residue had to be changed to murine to optimize the humanization. Position L46 is at the VL-VH interface, where it is buried and interacts directly with CDR-H3 (Fig. 2). The requirement for murine valine (as opposed to human leucine) implies that this residue plays an important role in the conformation of CDR-H3. The necessity of retaining LysH94 in VH, which is also adjacent to CDR-H3, suggests that CDR-H3 plays a major role in the binding of the antibody to VEGF.

The humanized version with optimal binding, 12-IgG1, exhibited only a 2-fold reduction in binding compared to the parent murine antibody (Table 3). An analysis of the binding kinetics of the humanized and chimeric F(ab)s showed that both had similar off-rates but that the humanized F(ab) had a 2-fold slower on-rate (Table 2), which accounts for the 2-fold reduction in binding. However, this modest reduction in on-rate did not result in any decreased ability to antagonize VEGF bioactivity. The two anti-

Table 3 *Binding of anti-VEGF IgG variants to VEGF*[a]

Variant	IgG1/chIgG1[b] Mean	SD	N
chIgG1	1.0		2
murIgG1[c]	0.759	0.001	2
12-IgG1[d]	1.71	0.03	2

[a] Anti-VEGF IgG variants were incubated with biotinylated VEGF and then transferred to ELISA plates coated with KDR-IgG (40).

[b] chIgG1 is chimeric IgG1 with murine VL and VH domains fused to human CL and IgG1 heavy chains; the EC_{50} for chIgG1 was 0.113 ± 0.013 μg/ml (0.75 nM).

[c] murIgG1 is muMAb VEGF A.4.6.1 purified from ascites.

[d] 12-IgG1 is F(ab)-12 VL and VH domains fused to human CL and IgG1 heavy chains.

bodies had essentially identical activity, both in an endothelial cell proliferation assay and in an *in vivo* tumor model.

Interestingly, an alternative approach using monovalent phage display has been also applied to the humanization of muMAb VEGF A.4.6.1. (49). Random mutagenesis of framework residues resulted in selection of variants with significantly improved affinity compared to the initial humanized MAb with no framework changes. However, the best variant obtained by this method had a less complete restoration of the binding affinity of muMAb VEGF A.4.6.1 compared to that reported in this study (49). Clearly, this does not rule out the possibility that other applications of phage display, such as affinity maturation of the CDRs (50), may result in variants with even higher affinity.

In conclusion, protein engineering techniques resulted in virtually complete acquisition by a human immunoglobulin framework of the binding properties and biological activities of a high-affinity murine anti-VEGF MAb. In view of the nearly ubiquitous up-regulation of VEGF mRNA in human tumors (12–16) and the ability of muMAb VEGF A.4.6.1 to inhibit the *in vivo* growth of a broad spectrum of tumor cell lines (20–23), VEGF is a major target of anticancer therapy. Clinical trials using rhuMAb VEGF should allow us to test the hypothesis that inhibition of VEGF-mediated angiogenesis is an effective strategy for the treatment of several solid tumors in humans. Such trials are already under way. Other important clinical applications of rhuMAb VEGF include the prevention of blindness secondary to proliferative diabetic retinopathy (17) or AMD (18). Clearly, the success of the humanization can be ultimately judged by the degree of anti-human globulin response and and by the clinical response in patients. However, the recent report of a Phase II study where rhuMAb HER2, a humanized MAb with the same framework as rhuMAb VEGF, did not induce any anti-globulin response in breast cancer patients and also demonstrated clinical efficacy (51), makes one optimistic. The results of this (51) as well as other (52) trials raise hope that, after many disappointing results (53), progress in antibody technology, coupled with selection of better targets, will bring therapy with MAbs closer to fulfilling its promises.

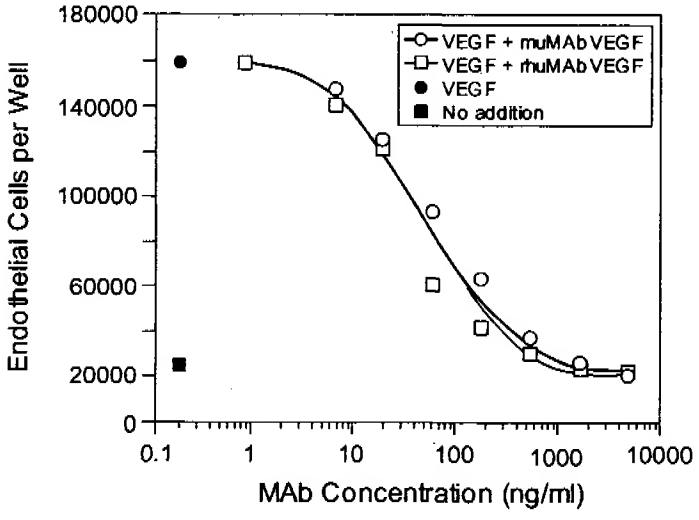


Fig. 3. Inhibition of VEGF-induced mitogenesis. Bovine adrenal cortex-derived capillary endothelial cells were seeded at the density of 6×10^3 cells/well in six-well plates, as described in "Materials and Methods." Either muMAb VEGF A.4.6.1 or rhuMAb VEGF (IgG1) was added at the indicated concentrations. After 2–3 h, $rhVEGF_{165}$ was added at the final concentration of 3 ng/ml. After 5 or 6 days, cells were trypsinized and counted. Values shown are means of duplicate determinations. The variation from the mean did not exceed 10%.

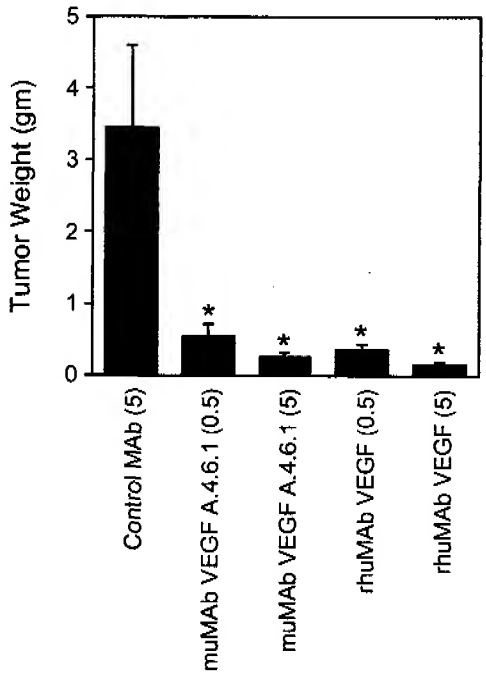


Fig. 4. Inhibition of tumor growth *in vivo*. A673 rhabdomyosarcoma cells were injected in BALB/c nude mice at the density of 2×10^6 per mouse. Starting 24 h after tumor cell inoculation, animals were injected with a control MAb, muMAb VEGF A.4.6.1, or rhuMAb VEGF (IgG1) twice weekly, i.p. The dose of the control MAb was 5 mg/kg; the anti-VEGF MAbs were given at 0.5 or 5 mg/kg, as indicated ($n = 10$). Four weeks after tumor cell injection, animals were euthanized, and tumors were removed and weighed. *, significant difference when compared to the control group by ANOVA ($P < 0.05$).

ACKNOWLEDGMENTS

We thank K. Garcia for performing the VEGF binding ELISA, W. Henzel for protein microsequencing, A. Padua for amino acid analysis, J. Bourell for mass spectrometry, and J. Silva for animal studies. We are grateful to the DNA synthesis and the DNA sequencing groups at Genentech. We also thank C. Adams, J. Kim, B. Fendly, B. Keyt, and M. Beresini for helpful comments and advice.

REFERENCES

1. Folkman, J., and Shing, Y. Angiogenesis. J. Biol. Chem., *267:* 10931–10934, 1992.
2. Klagsbrun, M., and D'Amore, P. A. Regulators of angiogenesis. Annu. Rev. Physiol., *53:* 217–239, 1991.
3. Garner, A. Vascular diseases. *In:* A. Garner and G. K. Klintworth (eds.), Pathobiology of Ocular Disease. A Dynamic Approach, Ed. 2, pp. 1625–1710. New York: Marcel Dekker, 1994.
4. Weidner, N., Semple, P., Welch, W., and Folkman, J. Tumor angiogenesis and metastasis. Correlation in invasive breast carcinoma. N. Engl. J. Med., *324:* 1–6, 1991.
5. Horak, E. R., Leek, R., Klenk, N., Lejeune, S., Smith, K., Stuart, M., Greenall, M., and Harris, A. Quantitative angiogenesis assessed by anti-PECAM antibodies: correlation with node metastasis and survival in breast cancer. Lancet, *340:* 1120–1124, 1992.
6. Macchiarini, P., Fontanini, G., Hardin, M. J., Squartini, F., and Angeletti, C. A. Relation of neovascularization to metastasis of non-small cell lung carcinoma. Lancet, *340:* 145–146, 1992.
7. Good, D., Polverini, P., Rastinejad, F., Beau, M., Lemons, R., Frazier, W., and Bouck, N. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc. Natl. Acad. Sci. USA, *87:* 6624–6628, 1990.
8. Clapp, C., Martial, J. A., Guzman, R. C., Rentier-Delrue, F., and Weiner, R. I. The 16-kilodalton N-terminal fragment of human prolactin is a potent inhibitor of angiogenesis. Endocrinology, *133:* 1292–1299, 1993.
9. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Mosem, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. Angiostatin. A novel angiogenesis inhibitor that mediates the suppression of metastasis by a Lewis lung carcinoma. Cell, *79:* 315–328, 1994.
10. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin. An endogenous inhibitor of angiogenesis and tumor growth. Cell, *88:* 277–285, 1996.
11. Ferrara, N., and Davis Smyth, T. The biology of vascular endothelial growth factor. Endocr. Rev., *18:* 4–25, 1997.

12. Berkman, R. A., Merrill, M. J., Reinhold, W. C., Monacci, W. T., Saxena, A., Clark, W. C., Robertson, J. T., Ali, I. U., and Oldfield, E. H. Expression of the vascular permeability/vascular endothelial growth factor gene in central nervous system neoplasms. J. Clin. Invest., *91:* 153–159, 1993.
13. Brown, L. F., Berse, B., Jackman, R. W., Guidi, A. J., Dvorak, H. F., Senger, D. R., Connolly, J. L., and Schnitt, S. J. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. Hum. Pathol., *26:* 86–91, 1995.
14. Brown, L. F., Berse, B., Jackman, R. W., Tognazzi, K., Manseau, E. J., Senger, D. R., and Dvorak, H. F. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. Cancer Res., *53:* 4727–4735, 1993.
15. Mattern, J., Koomagi, R., and Volm, M. Association of vascular endothelial growth factor expression with intratumoral microvessel density and tumour cell proliferation in human epidermoid lung carcinoma. Br. J. Cancer, *73:* 931–934, 1996.
16. Dvorak, H. F., Brown, L. F., Detmar, M., and Dvorak, A. M. Vascular permeability factor/vascular endothelial growth factor, microvascular permeability and angiogenesis. Am. J. Pathol., *146:* 1029–1039, 1995.
17. Aiello, L. P., Avery, R., Arrigg, R., Keyt, B., Jampel, H., Shah, S., Pasquale, L., Thieme, H., Iwamoto, M., Park, J. E., Nguyen, H., Aiello, L. M., Ferrara, N., and King, G. L. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N. Engl. J. Med., *331:* 1480–1487, 1994.
18. Lopez, P. F., Sippy, B. D., Lambert, H. M., Thach, A. B., and Hinton, D. R. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. Invest. Ophthalmol. Visual Sci., *37:* 855–868, 1996.
19. Kim, K. J., Li, B., Houck, K., Winer, J., and Ferrara, N. The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies. Growth Factors, *7:* 53–64, 1992.
20. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. Nature (Lond.), *362:* 841–844, 1993.
21. Warren, R. S., Yuan, H., Matli, M. R., Gillett, N. A., and Ferrara, N. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. J. Clin. Invest., *95:* 1789–1797, 1995.
22. Borgström, P., Hillan, K. J., Sriramarao, P., and Ferrara, N. Complete inhibition of angiogenesis and growth of microtumors by anti-vascular endothelial growth factor neutralizing antibodies. Novel concepts of angiostatic therapy from intravital videomicroscopy. Cancer Res., *56:* 4032–4039, 1996.
23. Melnyk, O., Schuman, M. A., and Kim, K. J. Vascular endothelial growth factor promotes tumor dissemination by a mechanism distinct from its effect on primary tumor growth. Cancer Res., *56:* 921–924, 1996.
24. Adamis, A. P., Shima, D. T., Tolentino, M., Gragoudas, E., Ferrara, N., Folkman, J., D'Amore, P. A., and Miller, J. W. Inhibition of VEGF prevents retinal ischemia-associated iris neovascularization in a primate. Arch. Ophthalmol., *114:* 66–71, 1996.
25. Miller, R. A., Oseroff, A. R., Stratte, P. T., and Levy, R. Monoclonal antibody therapeutic trials in seven patients with T-cell lymphoma. Blood, *62:* 988–995, 1983.
26. Schroff, R. W., Foon, K. A., Beatty, S. M., Odham, R. K., and Morgan, A. C., Jr. Human anti-murine immunoglobulin response in patients receiving monoclonal antibody therapy. Cancer Res., *45:* 879–885, 1985.
27. Neuberger, M. S., Williams, G. T., Mitchell, E. B., Jouhal, S. S., Flanagan, J. G., and Rabbits, T. H. A hapten-specific chimaeric IgE antibody with human physiological effector function. Nature (Lond.), *314:* 268–270, 1985.
28. Jones, P. T., Dear, P. H., Foote, J., Neuberger, M. S., and Winter, G. Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature (Lond.), *321:* 522–525, 1986.
29. Riechman, L., Clark, M., Waldmann, H., and Winter, G. Reshaping human antibodies for therapy. Nature (Lond.), *332:* 323–327, 1988.
30. Kabat, E. A., Wu, T. T., Perry, H. M., Gottesmann, K. S., and Foeller, C. Sequences of proteins of immunological interest, Ed. 5. Public Health Service, National Institutes of Health, Bethesda, MD, 1991.
31. Werther, W. A., Gonzalez, T. N., O'Connor, S. J., McCabe, S., Chan, B., Hotaling, T., Champe, M., Fox, J. A., Jardieu, P. M., Berman, P. W., and Presta, L. G. Humanization of an anti-lymphocyte function-associated antigen (LFA)-1 monoclonal antibody and reengineering of the humanized antibody for binding to rhesus LFA-1. J. Immunol., *157:* 4986–4995, 1996.
32. Carter, P., Presta, L., Gorman, C. M., Ridgway, J. B. B., Henner, D., Wong, W. L. T., Rowland, A. M., Kotts, C., Carver, M. E., and Shepard, H. M. Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc. Natl. Acad. Sci. USA, *89:* 4285–4289, 1992.
33. Eigenbrot, C., Randal, M., Presta, L., and Kossiakoff, A. A. X-ray structures of the antigen-binding domains from three variants of humanized anti-p185HER2 antibody 4D5 and comparison with molecular modeling. J. Mol. Biol., *229:* 969–995, 1993.
34. Kunkel, T. A. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA, *82:* 488–492, 1985.
35. Eaton, D. L., Wood, W. I., Eaton, D., Hass, P. E., Hollingshead, P., Wion, K., Mather, J., Lawn, R. M., Vehar, G. A., and Gorman, C. Construction, and characterization of an active factor VIII variant lacking the central one-third of the molecule. Biochemistry, *25:* 8343–8347, 1986.
36. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol., *36:* 59–74, 1977.
37. Gorman, C. M., Gies, D. R., and McCray, G. Transient production of proteins using an adenovirus transformed cell line. DNA Prot. Eng. Tech., *2:* 3–10, 1990.
38. Lucas, B. K., Giere, L. M., DeMarco, R. A., Chisholm, V., and Crowley, C. W. High-level production of recombinant proteins in CHO cells using a dicistronic DHFR intron expression vector. Nucleic Acids Res., *24:* 1774–1779, 1996.
39. Chisholm, V. High efficiency gene transfer in mammalian cells. *In:* D. M. Glover and B. D. Hames (eds.), DNA Cloning 4. Mammalian systems, pp. 1–41. Oxford: Oxford University Press, 1996.
40. Park, J. E., Chen, H., Winer, J., Houck, K. A., and Ferrara, N. Placenta growth factor. Potentiation of VEGF bioactivity, *in vitro* and *in vivo*, and high affinity binding to Flt-1 but not to Flk-1/KDR. J. Biol. Chem., *269:* 25646–25645, 1994.
41. Karlsson, R., Roos, H., Fagerstam, L., and Persson, B. Kinetic and concentration analysis using BIA technology. Methods: A Companion to Methods in Enzymology, Vol. 6, pp. 97–108, 1994.
42. Leung, D. W., Cachianes, G., Kuang, W-J., Goeddel, D. V., and Ferrara, N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science (Washington DC), *246:* 1306–1309, 1989.
43. Presta, L. G., Lahr, S. J., Shields, R. L., Porter, J. P., Gorman, C. M., Fendly, B. M., and Jardieu, P. M. Humanization of an antibody directed against IgE. J. Immunol., *151:* 2623–2632, 1993.
44. Eigenbrot, C., Gonzalez, T., Mayeda, J., Carter, P., Werther, W., Hotaling, T., Fox, J., and Kessler, J. X-ray structures of fragments from binding and nonbinding versions of a humanized anti-CD18 antibody: structural indications of the key role of VH residues 59 to 65. Proteins, *18:* 49–62, 1994.
45. Chothia, C., Lesk, A. M., Tramontano, A., Levitt, M., Smith-Gill, S. J., Air, G., Sheriff, S., Padlan, E. A., Davies, D., Tulip, W. R., Colman, P. M., Spinelli, S., Alzari, P. M., and Poljak, R. J. Conformations of immunoglobulin hypervariable regions. Nature (Lond.), *342:* 877–883, 1989.
46. Xiang, J., Sha, Y., Jia, Z., Prasad, L., and Delbaere, L. T. Framework residues 71 and 93 of the chimeric B72.3 antibody are major determinants of the conformation of heavy-chain hypervariable loops. J. Mol. Biol., *253:* 385–390, 1995.
47. Tramontano, A., Chothia, C., and Lesk, A. M. Framework residue 71 is a major determinant of the position and conformation of the second hypervariable region in the VH domains of immunoglobulins. J. Mol. Biol., *215:* 175–182, 1990.
48. Fischmann, T. O., Bentley, G. A., Bhat, T. N., Boulot, G., Mariuzza, R. A., Phillips, S. E. V., Tello, D., and Poljak, R. J. Crystallographic refinement of the three-dimensional structure of the FabD1.3-lysozyme complex at 2.5-Å resolution. J. Biol. Chem., *266:* 12915–12920, 1994.
49. Baca, M., Presta, L. G., O'Connor, S. J., and Wells, J. A. Antibody humanization using monovalent phage display. J. Biol. Chem., *272:* 10678–10684, 1997.
50. Barbas, C. F., III. Selection and evolution of high-affinity anti-viral antibodies. Trends Biotechnol., *14:* 230–234, 1996.
51. Baselga, J., Tripathy, D., Mendelshon, J., Baughman, S., Benz, C. C., Dantis, L., Sklarin, N. T., Deidman, A. D., Hudis, C. A., Moore, J., Rosen, P. P., Twaddel, T., Henderson, I. C., and Norton, L. Phase II study of weekly intravenous recombinant humanized anti-p185^{HER2} monoclonal antibody in patients with HER2/neu overexpressing metastatic breast cancer. J. Clin. Oncol., *14:* 737–744, 1996.
52. von Mehren, M., and Weiner, L. M. Monoclonal antibody-based therapy. Curr. Opin. Oncol., *8:* 493–498, 1996.
53. Riethmuller, G., Schneider-Gadicke, E., and Johnson, J. P. Monoclonal antibodies in cancer therapy. Curr. Opin. Immunol., *5:* 732–739, 1993.

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Biochimica et Biophysica Acta 1705 (2004) 69–89

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

http://www.elsevier.com/locate/bba

Review

The role of gelatinases in colorectal cancer progression and metastasis

Olaf R.F. Mook, Wilma M. Frederiks, Cornelis J.F. Van Noorden*

Academic Medical Center, University of Amsterdam, Department of Cell Biology and Histology, P.O. Box 22700, 1100 DE Amsterdam, The Netherlands

Available online 7 October 2004

Abstract

Various proteases are involved in cancer progression and metastasis. In particular, gelatinases, matrix metalloproteinase-2 (MMP-2) and MMP-9, have been implicated to play a role in colon cancer progression and metastasis in animal models and patients. In the present review, the clinical relevance and the prognostic value of messenger ribonucleic acid (mRNA) and protein expression and proenzyme activation of MMP-2 and MMP-9 are evaluated in relation to colorectal cancer. Expression of tissue inhibitors of MMPs (TIMPs) in relation with MMP expression in cancer tissues and the relevance of detection of plasma or serum levels of MMP-2 and/or MMP-9 and TIMPs for prognosis are also discussed. Furthermore, involvement of MMP-2 and MMP-9 in experimental models of colorectal cancer is reviewed. In vitro studies have suggested that gelatinase is expressed in cancer cells but animal models indicated that gelatinase expression in non-cancer cells in tumors contributes to cancer progression. In fact, interactions between cancer cells and host tissues have been shown to modulate gelatinase expression in host cells. Inhibition of gelatinases by synthetic MMP inhibitors has been considered to be an attractive approach to block cancer progression. However, despite promising results in animal models, clinical trials with MMP inhibitors have been disappointing so far. To obtain more insight in the (patho)physiological functions of gelatinases, regulation of MMP-2 and MMP-9 expression is discussed. Mitogen activated protein kinase (MAPK) signalling has been shown to be involved in regulation of gelatinase expression in both cancer cells and non-cancer cells. Expression can be triggered by a variety of stimuli including growth factors, cytokines and extracellular matrix (ECM) components. On the other hand, MMP-2 and MMP-9 activity regulates bioavailability and activity of growth factors and cytokines, affects the immune response and is involved in angiogenesis. Because of the multifunctionality of gelatinases, it is unpredictable at what stage of cancer development and in which processes gelatinase activity is involved. Therefore, it is concluded that the use of MMP inhibitors to treat cancer should be considered carefully.

Keywords: Gelatinase; Colorectal cancer; Tumor progression; Metastasis; Clinical; Experimental model

Contents

* Corresponding author. Department of Cell Biology and Histology, AMC-L 3/111, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. Tel.: +31 20 566 4970/4966; fax: +31 20 697 4156.

E-mail address: c.j.vannoorden@amc.uva.nl (C.J.F. Van Noorden).

0304-419X/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbcan.2004.09.006

1. Introduction

With few exceptions, death of colorectal cancer patients is caused by metastatic disease rather than the primary tumor [1]. The process by which malignant cells escape from the primary tumor and develop tumors at a secondary site is called the metastatic cascade. Key events in the cascade are generally considered to be invasion of stroma, intravasation of the circulatory system at the primary site, extravasation at the secondary site and outgrowth of new tumors. These processes require degradation of extracellular matrix (ECM) components by proteolytic enzymes [2]. Various types of proteinases have been found to participate in ECM turnover, but matrix metalloproteinases (MMPs) are the principal ECM-degrading enzymes. MMPs have historically been categorized in four subgroups on the basis of their specificity for ECM components: collagenases, gelatinases, stromelysins and matrilysins. As the list of MMPs is growing, a numbering system is adapted and MMPs are classified now according to their structure. Eight structural classes are recognized, five classes of MMPs being secreted and three being membrane-type MMPs (MT-MMPs; Table 1). Matrix degradation is a normal physiological process involved in embryonic development, organ morphogenesis, blastocyst implantation, ovulation, hair follicle growth, bone remodelling and wound healing [3]. Up-regulation of MMP expression has been implicated in various diseases including arthritis, artherosclerosis and tumor progression and metastasis [4].

One particular group of MMPs, the gelatinases A and B (structure group: gelatin-binding) also known as 72- and 92-kDa type IV collagenase or MMP-2 and MMP-9, respectively, are of particular interest with respect to the development and progression of colorectal cancer. Historically, interest in these enzymes was based on the capacity of these enzymes to degrade type IV collagen, a major component of basement membranes. Degradation of basement membranes is considered to be essential in invasive growth and metastasis. However, we now know many more biological functions of gelatinases. It has been shown that they are also involved in cell differentiation, apoptosis, angiogenesis, immune surveillance and cancer cell growth [5].

Gelatinases are expressed and secreted as inactive proenzymes. Expression is regulated by growth factors, cytokines, cell–matrix and cell–cell interactions. Enzyme activity is regulated extracellularly and its regulation is mainly based on the balance between proenzyme activation and inhibition by tissue inhibitors of MMPs (TIMPs) of which four are known at present (TIMP-1–4). TIMP-1, -2 and -4 are soluble proteins whereas TIMP-3 is matrix-associated. TIMP-1 and TIMP-2 are endogenous inhibitors of all types of active MMPs whereas TIMP-3 and TIMP-4 are inhibitors of most MMPs. Furthermore, TIMP-1 forms a specific complex with pro-MMP-9 and this complex formation inhibits activation of pro-MMP-9. TIMP-2 also complexes with pro-MMP-2. Low TIMP-2 levels are associated with MT1-MMP-mediated MMP-2 activation, whereas high TIMP-2 levels directly inhibit MT1-MMP-mediated MMP-2 activation [6]. Furthermore, TIMPs also have other functions besides inhibition of MMPs. For example, TIMPs have been shown to induce or inhibit apoptosis in various cell types [7].

Gelatinases have been reported to be associated with invasive and metastatic behavior of malignant tumors on the basis of their elevated expression in relation with invasion and metastasis. Tumors consist of cancer cells and stroma. Although cancer cells may be the source of gelatinases in some cases, in the majority of cases only stromal cells express gelatinases [8,9]. However, elevated mRNA or protein levels in tumors are not necessarily linked with invasion and metastasis because these are not per se biologically or pathologically relevant. Only active forms of MMP-2 and MMP-9 are.

In the present review, recent studies are discussed on the clinical significance of gelatinase expression in combination with TIMP expression in colorectal cancer

Table 1
Classes of MMPs and MMP nomenclature

Structural class	Names and synonyms	MMP nomenclature
Secreted MMPs		
Minimal domain	**Matrilysin**, matrin, PUMP1, small uterine metalloproteinase	MMP-7
	Matrilysin 2, endometase	MMP-26
Simple hemopexin domain	**Collagenase**-1, interstitial collagenase, fibroblast collagenase, tissue collagenase	MMP-1
	Collagenase-2, neutrophil collagenase, PMN collagenase, granulocyte collagenase	MMP-8
	Collagenase 3	MMP-13
	Stromelysin 1, transin-1, proteoglycanase, pro-collagenase activating protein	MMP-3
	Stromelysin 2, transin-2	MMP-10
	Metalloelastase, macrophage elastase, macrophage metalloelastase	MMP-12
	Collagenase-4 (no human homologue)	MMP-18
	RASI-1, MMP-18[a]	MMP-19
	Enamelysin	MMP-20
	None	MMP-27
Furin activated and secreted	**Stromelysin** 3	MMP-11
	Epilysin	MMP-28
Gelatin-binding	**Gelatinase** A, 72-kDa gelatinase, 72-kDa type IV collagenase, neutrophil gelatinase	MMP-2
	Gelatinase B, 92-kDa gelatinase, 92-kDa type IV collagenase	MMP-9
Vitronectin-like insert	Homologue of *Xenopus* XMMP	MMP-21
MT-MMPs		
Transmembrane	MT1-MMP	MMP-14
	MT2-MMP	MMP-15
	MT3-MMP	MMP-16
	MT5-MMP	MMP-24
GPI-linked	MT4-MMP	MMP-17
	MT6-MMP, leukolysin	MMP-25
Type II transmembrane	Cysteine array MMP (CA-MMP), femalysin, MIFR, MMP-21/MMP-22	MMP-23

Historical names **in bold**. Adapted from Egeblad and Werb, Nature Rev 2002:21,161.

[a] When MMP-19 was cloned it was called MMP-18. However, an MMP from *Xenopus* had already received that name and therefore this MMP is now called MMP-19.

and metastasis. We also evaluate animal studies and in vitro studies on the role of gelatinases in colon cancer and metastasis.

2. Clinical relevance of the correlation between gelatinase expression and tumor progression

2.1. Expression at the mRNA level

Levels of mRNA have been detected by in situ hybridization and polymerase chain reaction (PCR), protein levels by immunohistochemistry and Western blot and activity levels by gelatin zymography and hydrolysis of quenched fluorescent substrate. It is particularly the latter approach that provides information on functional involvement of gelatinases in colon cancer. Expression of MMPs in general and of gelatinases in particular has been extensively studied in biopsies and resection material of primary tumors and metastases of colon cancer patients. MMP-2 mRNA levels have been found to be overexpressed in carcinomas [10–13]. Overexpression of MMP-2 mRNA has been found in all Dukes' stages and expression levels did not correlate with the stages in the study of Heslin [14], whereas Collins et al. [12] showed a significant increase of MMP-2 mRNA levels in Dukes' stages B and C whereas mRNA levels of MMP-9, MT1-MMP, TIMP-1 and TIMP-2 remained unaltered. As a consequence, the MMP-2/TIMP-2 ratio was found to be increased in Dukes' stages B and C as compared with normal mucosa. Similar findings with respect to levels of mRNA of MMP-2, MT1-MMP and TIMP-2 were reported by Ornstein and Cohn [13]. On the other hand, MMP-2 mRNA levels were increased in all stages of colorectal carcinomas, whereas TIMP-2 mRNA levels were decreased in Dukes' stages C and D in the study of Chan et al. [11]. As a consequence, the MMP-2/TIMP-2 ratio was also elevated in Dukes' stages C and D. This shows that increased MMP-2/TIMP-2 ratios can be a result of either increased MMP-2 expression or decreased TIMP-2 expression.

In line with the observations of Collins et al. [12], MMP-2 mRNA was significantly more often present in tumor lesions than in normal colon tissue whereas MMP-9 mRNA expression was not significantly different in tumors of the colon and healthy mucosa in the study of Masuda and Aoki [15]. Also in this study, TIMP-1 and TIMP-2 mRNA expression was similar in normal colon and colon cancer. However, a relationship between MMP-2 mRNA levels and Dukes' stage was not found. Patients with or without metastases did not show any difference in expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 mRNA [15], but interestingly, MMP-2 mRNA expression in normal colon

tissue of these patients was correlated with the presence of metastases (Dukes' D). It was concluded that prognosis of colorectal cancer patients is possible on the basis of expression patterns of MMP-2 and TIMP-2 mRNA in normal colon tissue [15]. In conclusion, MMP-2 has been shown to be overexpressed in colorectal cancer. However, clear correlations between MMP-2 expression levels and Dukes' stage have only occasionally been reported. On the other hand, an increased ratio of MMP-2 and TIMP-2 has been shown more frequently in cancer and it seems that an increased MMP-2/TIMP-2 ratio correlates with Dukes' stage.

Besides the studies of Collins et al. [12] and Masuda and Aoki [15] that did not show a link between MMP-9 expression and colorectal cancer progression, MMP-9 mRNA was detected in half of the colorectal tumors investigated, whereas a significantly higher incidence was found when liver metastases were present indicating that MMP-9 expression is correlated with tumor progression [16]. Furthermore, elevated MMP-9 mRNA levels in colorectal cancer tissues as compared with healthy colon tissue indicated a shorter disease-free period and a shorter overall survival [9,17]. Roeb et al. [18] also showed that MMP-9 mRNA levels were elevated in colon carcinomas but not in rectal carcinomas whereas TIMP-1 mRNA levels remained unchanged. Heslin et al. [14] found that MMP-9 was overexpressed in all Dukes' stages but a correlation between MMP-9 expression and Dukes' stage was not apparent.

In conclusion, elevated levels of mRNA of MMP-2 or MMP-9 in colorectal cancer tissues as compared with healthy mucosa suggests involvement of these enzymes in cancer progression, but a correlation between MMP-2 or MMP-9 mRNA levels and Dukes' stage has only been reported incidentally.

Levels of TIMP-3 mRNA have been found to be increased in colorectal cancer, but protein levels of TIMP-3 were decreased in correlation with Dukes' staging [19]. In the study of Powe et al. [20], it was concluded that loss of TIMP-3 mRNA expression may contribute to enhanced cancer progression. Although it has been shown that TIMP-1 and TIMP-2 mRNA expression remained unaltered or TIMP-2 mRNA expression was down-regulated in advanced stages of cancer, overexpression of TIMP-1 mRNA was found to correlate with lymph node metastasis and distant metastasis [21–23] and elevated TIMP-2 mRNA expression was found to be related with clinical staging from dysplasia to adenocarcinoma [24,25]. These findings suggest that besides inhibition of MMPs by TIMP-1 and TIMP-2 in relation to carcinogenesis, overexpression may also induce cancer cell growth and development, due to other functions of TIMPs and in particular their growth factor-like properties [26]. However, it should be realized that translation of mRNAs into proteins of MMPs and TIMPs is also regulated so that protein levels may be a better indicator for the role of these proteinases and their inhibitors in invasion and metastasis.

2.2. Expression at the protein level

Tissue MMP-2 protein levels increase as polyps progress to adenocarcinoma [27] and increased levels of MMP-2 protein have been observed in invasive regions of colorectal tumors [28]. Protein levels of MMP-2 in colorectal cancer correlated significantly with Dukes' staging in the study of Levy et al. [10]. MMP-2 protein was significantly increased in Dukes' D whereas TIMP-2 protein levels were decreased in Dukes' C and Dukes' D tumors in the study of Chan et al. [11]. As a result, the ratio between MMP-2 and TIMP-2 was increased in Dukes' C and Dukes' D tumors [11]. Furthermore, Kim and Kim [29] found a positive correlation between MMP-2 protein levels and both Dukes' staging and angiogenesis, whereas a positive correlation between both MMP-2 and MMP-9 protein levels and Dukes' staging has been found as well [30]. In a small set of colorectal cancer samples, the absence of type IV collagen was related with the presence of MMP-9. Absence of type IV collagen has been found in all patients with colorectal cancer that had metastases, and only in a quarter of the patients without metastases [31]. Immunolocalization showed that MMP-9 is more frequently expressed in advanced tumors and particularly in invasive tumor regions, where cancer cells are in close proximity of inflammatory cells, suggesting that localized proteolytic activity contributes to invasion [32]. On the other hand, the number of MMP-9- and urokinase-type plasminogen activator receptor (uPAR)-positive cells was also found to be inversely correlated with metastasis of colon cancer, suggesting that MMP-9 and uPAR can have a dual role. They can both promote cancer development and be involved in host defence [33]. In contrast, Ring et al. [34] observed that the presence of MMP-2 and MMP-9 protein did not correlate with Dukes' staging or survival time. On the other hand, TIMP-2 was more often present at basement membranes and/or subglandular structures in primary colorectal tumors of patients without metastases than in those of patients with metastases [34]. It should be noted that only tumors of different Dukes' stages were compared in this study and not expression levels in tumors in comparison with normal mucosa. Nevertheless, it was demonstrated that TIMP-2 staining correlated negatively with metastasis. So, despite the fact that MMP-2 and MMP-9 staining did not correlate with tumor stage in this study, the study indicated that the MMP2/TIMP-2 protein ratios may be in favour of proteolysis in advanced stages.

On the other hand, elevated TIMP-1 expression but not TIMP-2 expression correlated positively with Dukes' staging and negatively with survival in the study of Joo et al. [35]. Therefore, it was hypothesised that TIMP-1 expression may be growth-promoting as well. However, MMP-2 and MMP-9 protein expression was not evaluated in this study. This may be crucial for understanding the

physiological role of TIMP-1 because Baker et al. [36] showed significantly higher TIMP-1 levels in tumors whereas overall MMP activity was also higher in these tumor tissues (see below). This indicates that determination of TIMP-1 protein levels is only informative when linked with levels of MMPs.

In conclusion, MMP-2 and MMP-9 protein levels in colorectal tumors are elevated as compared to normal mucosa. In general, correlations have been found between MMP-2 and MMP-9 protein levels and metastatis of colorectal cancer, whereas both decreased and elevated TIMP protein levels have been found in relation with metastasis in colorectal cancer patients.

2.3. Expression at the activity level

The functional status of MMP-2 and MMP-9 can be obtained by studying activity of these proteinases. Activation of the proenzymes has been studied with SDS-PAGE gelatin zymography. Lysis bands of active MMP-2, and not of active MMP-9, have been found in gelatin zymograms by Yagamata et al. [37] and Emmert-Buck et al. [28] in colon cancer specimens in comparison with healthy colon tissue. Activation of MMP-2 was considered to be a feature of the malignant phenotype of colorectal cancers [38] because proenzyme activation of MMP-2 was elevated in Dukes' D colorectal cancer. Activation of both MMP-2 and MMP-9 has been found in colon carcinomas in comparison with healthy mucosa [18,39]. Furthermore, the active form of MMP-2 and MMP-9 was predominantly present in patients with metastases [31]. In the study of Baker et al. [30], gelatin zymography showed that colorectal cancer tissues express significantly more often active MMP-2 and MMP-9 as compared with normal mucosa. Levels of inactive and active MMP-9 but not of active MMP-2 correlated with Dukes' staging [30]. Waas et al. [40] reported that absolute levels of active MMP-2, but not of active MMP-9, were significantly lower in stage D tumors than in stage B tumors. Ratios of active and inactive MMP-2 were increased fivefold in tumors as compared with normal mucosa, whereas the ratio for MMP-9 was largely decreased in tumor tissues [40].

Gelatin zymography discriminates between inactive and active MMPs but does not allow a distinction between free MMPs and those complexed with natural inhibitors. Therefore, absolute values of gelatinolytic activity cannot be obtained in this way. Fluorogenic substrate hydrolysis provides quantitative values of gelatinolytic activity. Baker et al. [36] showed with a quenched fluorescent substrate that gelatinolytic activity was higher in tumor homogenates than in normal tissue homogenates and higher in homogenates of carcinomas than of adenomas. A correlation between gelatinolytic activity and Dukes' staging was not found but T3 and T4 tumors showed a higher activity than T1 and T2 tumors. Waas et al. [40] showed that gelatinolytic activity as determined by substrate hydrolysis was significantly higher in homogenates of colorectal tumors and at the transition of cancer tissue and healthy tissue as compared with homogenates of normal colorectal tissues. However, the activity did not correlate with any clinicopathological parameter including T staging, which was in contrast with zymographic findings on MMP-2. Only homogenates of transitional tissue of patients with metastases showed higher activity as compared with those tissues of patients without metastases [40].

In conclusion, proenzyme activation of MMP-2 and MMP-9 is in general increased in colorectal tumors as compared with healthy mucosa. For both active MMP-2 and active MMP-9, correlations have been shown with Dukes' staging. Therefore, MMP-2 and MMP-9 are considered to be involved in the malignant phenotype of colorectal cancer. Gelatinolytic activity is elevated in tumors compared with healthy tissue. However, gelatinolytic activity within the tumor does not correlate with Dukes' staging but it has been shown that it rather represents the progression of the tumor. Furthermore, elevated gelatinolytic activity in transitional tissue has been linked with the presence of metastases.

2.4. Gelatinases in plasma and serum

Because MMPs are regularly overexpressed in colorectal tumors and may be released into the circulation, MMP levels in serum or plasma of cancer patients have been evaluated as a prognostic tool. A relation was shown between levels of MMP-9 in plasma of patients and the presence of colorectal cancer, but plasma levels of MMP-9 did not enable discrimination between metastatic and non-metastatic disease [41,42]. Later, it was reported that elevated plasma levels of either MMP-9 or MMP-9/TIMP complexes correlate with reduced survival of Dukes' D patients [43]. In contrast, MMP-2 levels in serum [42] or plasma [44] of colorectal cancer patients were not found to be elevated. Furthermore, Pucci-Minafra et al. [39] demonstrated that pro-MMP-9 levels but not pro-MMP-2 levels in serum were associated with cancer. Lysis bands at 220, 200 and 116 kDa were observed in serum of cancer patients and not in serum of healthy individuals. The 200- and 116-kDa bands have been interpreted as pro-MMP-9/TIMP-1 complexes and the 220-kDa band as a dimer of pro-MMP-9. Activated MMP-2 and MMP-9 were never found in sera of both cancer patients and healthy individuals, despite the fact that active MMP-2 and MMP-9 were present in tumors. These differences in gelatinolytic patterns in sera and tumor homogenates suggest that gelatinolytic activity in blood of cancer patients is not predictive for gelatinolytic activity in the tumor.

Elevated pre-operative TIMP-1 plasma levels have been found to be correlated with poor prognosis [45] and with Dukes' stage D of colorectal cancer [46], which is in line with high levels of TIMP-1 mRNA and protein that have

been found in colorectal tumors. Furthermore, Yukawa et al. [47] found that elevated TIMP-1 plasma levels predicted invasion of the serosa and Dukes' C and D stages in two thirds of colorectal cancer patients. On the other hand, significant differences in TIMP-1 levels were not found by Oberg et al. [48] in Dukes' A–C stages versus controls. A combination of TIMP-1 serum levels with carcinoembryonic antigens (CEA) levels increased the prognostic value over that of TIMP-1 levels alone. When protein levels of CEA and not of TIMP-1 were increased, it was an indication of lymph node invasion, whereas elevated serum levels of both CEA and TIMP-1 indicated that metastases were present [49,50].

Another approach to determine the status of disease of patients was introduced by Okada et al. [51]. They collected bile of patients with and without liver metastases for the detection of inactive and active forms of MMP-2 and MMP-9. Active MMP-2 appeared to be significantly elevated in bile of patients with metastases as compared with patients without metastases, whereas levels of pro-MMP-2 were similar. On the other hand, inactive MMP-9 was significantly higher in patients with liver metastases whereas active MMP-9 was not detected. It was suggested that active MMP-2 and pro-MMP-9 may be useful markers for predicting liver metastasis in colorectal cancer.

In conclusion, the involvement of MMP-2 or MMP-9 or both in colorectal cancer has been demonstrated more convincingly at the protein level than at the mRNA level. Correlations have been found between MMP-2 and MMP-9 protein levels and Dukes' staging whereas activation of pro-MMP-2 and pro-MMP-9 seem to be key events in tumor progression. Detection of TIMP-1 and TIMP-2 levels increases the discriminating power because ratios of MMPs and their inhibitors provide clues for the net proteolytic capacity. However, TIMP-1 and TIMP-2 expression has been associated with tumor progression as well, probably due to their growth factor-like properties [26]. Quenched substrate hydrolysis by tumor homogenates represents net gelatinolytic activity. Net gelatinolytic activity is elevated in carcinomas but differentiation between Dukes' stages on the basis of gelatinolytic activity does not seem to be possible. Interestingly, depth of invasion of the tumor is sometimes correlated with elevated gelatinolytic activity. Attempts have been made to predict tumor stages on the basis of plasma or serum levels of MMP-2 and/or MMP-9 but so far with little success. TIMP-1 plasma levels in combination with CEA levels were shown to be more powerful in prognosis.

3. Experimental studies

3.1. Gelatinase expression in colon cancer cells

Metastatic capacity of colorectal cancer cells and elevated levels of gelatinases in tumors have often been considered to be due to enhanced MMP production by the malignant cells [52]. This relationship between elevated metastatic capacity and increased MMP expression has been shown indirectly in in vitro experiments. For example, serum-free culture media of poorly metastatic and highly metastatic murine colon carcinoma cells both contained MMP-2 and MMP-9, but the amounts of MMP-2 were markedly higher in the highly metastatic cell line [53,54]. Kishi et al. [55] examined TIMP-1 and TIMP-2 expression in poorly metastatic (NM11) and highly metastatic (LuM1) murine colon carcinoma cells. They found that poorly metastatic cells secreted lower levels of gelatinases and higher levels of TIMP-1 and TIMP-2 than highly metastatic cells. The balance between gelatinases and inhibitors in the poorly metastatic cell line was in favour of the inhibitors whereas the balance was in favour of proteolysis in the highly metastatic cell line. Hyuga et al. [56] used the same cell lines and showed that an autocrine factor was responsible for the up-regulation of MMP-9 (but not of MMP-2) in the highly metastatic cell line. Hewitt et al. [57] showed that a more tumorigenic colon cancer cell line (SW620) expressed lower levels of TIMP-2 and TIMP-3 than a less tumorigenic cell line (SW480). This again indicates that a balance in favour of proteolysis promotes invasion. Surprisingly, a tenfold increased expression of TIMP-1 was found in the more tumorigenic cell line as well [57]. This is in line with the findings that increased levels of TIMP-1 in colorectal cancers and serum of colorectal cancer patients are an indicator for poor prognosis [35,45,46].

MMPs and TIMPs expressed by cancer cells may be important in invasion and metastasis. However, studies have shown that cultured cancer cells which do not express MMP-2 and MMP-9 can be metastatic in vivo as well [58,59]. Tumors developed in vivo contained substantial amounts of MMP-2 and MMP-9, which were produced by stromal cells such as fibroblasts, monocytes and macrophages. Cancer cell–host cell interactions and the generation of a tumor-specific microenvironment for the regulation of MMP expression in host cells are appreciated as important factors in metastasis. Therefore, MMPs and TIMPs may be important even when cancer cells do not produce these proteins themselves.

3.2. Gelatinase expression in non-cancer cells

Tumors consist of cancer cells and non-cancer cells. There is a large contribution of non-cancer cells in gelatinase expression in tumors. Gelatinases are expressed by fibroblasts and by a variety of immune cells including macrophages, monocytes, dendritic cells, natural killer cells, lymphocytes and neutrophils (reviewed in Opdenakker et al. [60]).

MMP-2 mRNA in colon cancer tumors is mainly localized in fibroblasts whereas MMP-9 mRNA is mainly expressed in tissue macrophages. Thus, it seems that

MMP-2 and MMP-9 in colorectal tumors are mainly produced by fibroblasts and macrophages, respectively. However, a number of other cells involved in tumor biology have been found to express MMP-2 and MMP-9. For example, migration of monocytes is partly inhibited by doxazosin due to inhibition of MMP-9 activity [61]. Monocyte-derived dendritic cells (DC) activate naïve T-cells into cytotoxic T-cells (CTL) which are essential to initiate the specific immune response. DC, inoculated with colon cancer cell total RNA, showed an increased anti-tumor CTL response in vitro [62]. Stimulated DC showed increased migration through matrigel mainly due to increased MMP-9 expression and decreased TIMP-1 and TIMP-2 expression [63]. Furthermore, migration of natural killer (NK) cells into tumor metastases is a prerequisite for their cytolytic function in cancer. NK cells have been reported to express MMP-2 and MMP-9 and degrade matrigel in an interleukin-2 (IL-2)-dependent manner [64]. MMP-9 is expressed constitutively in resting T-cells, whereas MMP-2 expression is induced and MMP-9 expression is elevated upon T-cell activation [65]. Epstein–Barr virus-immortalized B-cells showed increased MMP-9 expression upon IL-1β and IL-8 treatment [66]. IL-8 causes the immediate release of MMP-9 from neutrophils [67] and a direct role of MMP-9 in migration of neutrophils was shown in MMP-9 knock-out mice where migration was significantly impaired in young mice. However, these effects were much less pronounced in adult mice and apparently the lack of MMP-9 was compensated for [68].

Therefore, gelatinase expression in tumors is not necessarily a consequence of gelatinase expression in cancer cells. Many, if not all, immune cells express either MMP-2 or MMP-9 or both. Their primary function is probably related with migration of immune cells. Cancer cells may use host cell gelatinases for their own purpose. To do so, colon cancer cells can modulate MMP expression in host cells and, in combination with dampening of the immune response (see below), use host-derived gelatinases for their own advantage.

3.3. Interaction of colon cancer with host cells

Cultured colorectal cancer cells were found to induce MMP-9 expression in fibroblasts via direct cell–cell contact. In contrast, cancer cells derived from liver metastases and lymph node metastases did not induce MMP-9 expression in fibroblasts [69]. Fibroblasts, cultured in the presence of MMP-2-negative metastatic colorectal cancer cells (TF-43C), were stimulated to produce pro-MMP-2 but not when cocultured with non-metastatic cells (TF-17C). Furthermore, conditioned medium of metastatic cancer cells can induce similar effects, showing that soluble factors secreted by cancer cells can induce MMP-2 expression in fibroblasts [59]. On the other hand, cancer cells or conditioned medium did not induce MMP-2 production in fibroblasts in the study of Ko et al. [70]. These contradictory findings suggest that either fibroblasts behave differently in their response to cancer cells or that colon cancer cells differ with respect to their effects on fibroblasts. The first hypothesis was confirmed by Fabra et al. [71] who showed that cocultures of an invasive and metastatic colon cancer cell line KM12SM and either colon or lung fibroblasts produced significant amounts of pro-MMP-2 and active MMP-2 in the medium, whereas cocultures with skin fibroblasts did not.

Coculture of monocytes with MMP-2- and MMP-9-negative metastatic colon cancer cells (SW620-S5) induced secretion of moderate levels of MMP-9 and high levels of MMP-2. Conditioned media of these cancer cells induced similar responses in monocytes, implicating a paracrine regulation. Cocultures of monocytes with non-metastatic cancer cells (SW620-P) reduced MMP-9 secretion, whereas MMP-2 secretion was unaffected [72]. These studies clearly show that metastatic colon cancer cells are able to induce MMP expression and/or MMP secretion in stromal cells, either via direct contact or via paracrine regulation.

4. Animal studies

The in vitro findings described above have been confirmed by in vivo experiments. Orthotopically implanted tumors on the cecum of recipient mice derived from MMP-2- and MMP-9-negative human colorectal cancer cell lines with different metastatic potentials (SW480, SW620 and SW620S5, respectively) expressed significant amounts of MMP-2 and MMP-9. MMP-9 was host-derived and was expressed by stromal cells. These findings strongly suggest that not cancer cells but stromal cells are the major source of MMP-2 and MMP-9 and that metastatic cancer cells are able to up-regulate MMPs in host cells in vivo [58]. Cancer cell–host cell interactions may even be more important than MMP expression by cancer cells themselves since MMP-2-expressing cancer cells (TF-17C) have been found not to be tumorigenic whereas colon cancer cells (TF-43C), which do not produce MMP-2, can be both tumorigenic and metastatic [59]. Furthermore, intraportal injection of cultured colon cancer cells (CC531s) that do not produce MMP-2 and MMP-9 results in liver metastases that contain considerable amounts of MMP-2 and MMP-9 [73].

4.1. Natural inhibitors in animal models

The importance of MMPs in cancer progression and metastasis has been established by application of inhibitors of MMPs in animal models. Introduction of a functional TIMP-3 gene in a TIMP-3-deficient human colon cancer cell line (DLD-1) decreased its growth potential, and the

Table 2
Application of natural inhibitors and synthetic small-molecular MMP inhibitors in experimental models of colon cancer, their specificity and their effect

Inhibitor	Specificity	Type of cancer cell	Effects	Refs
BB-94 (batimastat)	Broad range	C170HM2	From day 10 (after ip administration of cancer cells): liver metastases ↓, diameter ↓	[80]
BB-94 (batimastat)	Broad range	DHD/K12	From day 2: prevention of peritoneal carcinomatosis and liver metastases. From day 13: peritoneal carcinomatosis ↓, number liver metastases ↓	[79]
BB-94 (batimastat)	Broad range	C170HM2	From day 0 and day 10: peritoneal carcinomatosis ↓, ascites (day 0) ↓, ascites (day 10) =	[198]
BB-94 (batimastat)	Broad range	AC 1935[a]	From day 7: orthotopic primary tumors ↓, local and regional invasion ↓, distant metastases ↓	[78]
BB-2516 (marimastat)	Broad range	C-1H	Colonization: metastases =	[88]
MMI-166	MMP-2. 9, 14	TK-4	Orthotopic primary tumors =, liver metastases: incidence ↓, diameter ↓	[86]
MMI-166	MMP-2, 9, 14	C-1H	Liver colonization: metastases ↓	[88]
MMI-166	MMP-2, 9, 14	TK-4	Orthotopic primary tumors =, liver metastases ↓, angiogenesis ↓, synergistic effect with mitomycin C.	[89]
MMI-166	MMP-2, 9, 14	C-1H	Liver colonization: metastases ↓, synergistic effect with CPT-11	[90]
AG-3340	Broad range	C-1H	Liver colonization: metastases ↓	[88]
KB-R7785	MMP-1, 3, 9	C-26	Primary tumors (dorsal skinfold chamber) ↓, angiogenesis ↓, lung metastases: number ↓, diameter ↓	[85]
CT1746	MMP-2, 3, 9	Co-3	Orthotopic primary tumors ↓, tumor spread ↓, metastases ↓	[82]
BAY12-9566	MMP-2, 3, 9	?	Orthotopic primary tumors ↓, distant metastases ↓, diameter ↓	[83]
R-94138	MMP-2, 9	HT-29, LS174T	Lymph node metastases ↓	[84]
BPHA	MMP-2, 9, 14	C-1H	Liver colonization ↓	[87]
TIMP-1	MMP-9	KM12SM	TIMP-1 expression in cancer cells: orthotopic primary tumors ↓, liver metastases ↓, rTIMP-1 (ip): primary tumor =, liver metastases =	[77]
TIMP-2	MMP-2	C51	TIMP-2 expression in cancer cells: subcutaneous tumor volume ↓, TIMP-2 adenoviral injection in preestablished tumors: tumor volume ↓, angiogenesis =, apoptosis ↑	[75]
TIMP-2	MMP-2	LS174T	TIMP-2 expression in liver prior to cancer cell administration: metastases ↓	[76]
TIMP-2	MMP-2	LS174T	TIMP-2 expression in liver: growth established metastases ↓	[76]
TIMP-3		DLD-1	TIMP-3 expression in cancer cells: growth potential ↓	[74]

(=) No effect; (↑) increased; (↓) decreased.
[a] AC 1935 is a fifth passage primary colon adenocacinoma obtained from patient AC 1935.

reduction was related with the amount of TIMP-3 that was expressed [74]. Transfection of cancer cells or endothelial cells with the TIMP-2 gene resulted in inhibited invasion of these cells in matrigel and 50% inhibition of tumor development in mice treated with TIMP-2-transfected cancer cells. Growth rates of established tumors were significantly reduced by a single local injection with a TIMP-2 gene-containing adenovirus [75]. Interestingly, Brand et al. [76] showed that overexpression of TIMP-2 in hepatocytes reduced metastasis in the liver by 95% after cancer cell (LS 174T) inoculation. Furthermore, transfection of the TIMP-2 gene to livers with preexisting metastases resulted in 80% reduction in tumor growth [76]. Growth of primary tumors of orthotopically-implanted colon cancer cells (KM12SM) and development of liver metastases in nude mice were inhibited when the colon cancer cells were transfected with the TIMP-1 gene [77]. These data clearly show that overexpression of natural inhibitors of MMPs in cancer cells or host cells can inhibit experimental colon cancer growth and metastasis (Table 2), whereas an inducing effect of either TIMP-1 or TIMP-2 has not yet been described in animal models.

4.2. Synthetic inhibitors in animal models

In analogy with endogenous MMP inhibitors, administration of synthetic MMP inhibitors affects colon cancer progression and metastasis in animal models. Batimastat (British Biotech; BB-94), a first-generation broad-range MMP inhibitor, reduced growth of orthotopically implanted primary colon tumors, local invasion and distant metastases [78]. Liver metastasis was completely prevented when treatment with batimastat was started at the time of intraperitoneal injection of cancer cells, whereas treatment that was started at 13 days after administration of cancer cells did not prevent liver metastasis but resulted in reduced liver metastasis and prolonged survival [79]. Treatment with batimastat that was started at 10 days after inoculation of cancer cells (C170HM2) significantly reduced both numbers

and diameters of liver metastases [80]. On the other hand, batimastat introduced liver metastases in an animal model of breast cancer and induced liver metastases derived from a T-cell lymphoma. Furthermore, daily administration of batimastat to healthy animals induced MMP-9 expression and increased expression of MMP-2 in liver tissue [81]. In conclusion, broad-range inhibition of MMPs in animal models of colon cancer metastasis has shown the involvement of MMPs in colon cancer progression and metastasis. Early MMP inhibition inhibits primary tumor growth and metastasis whereas this effect on established metastases is less pronounced (Table 2).

Clinical trials with batimastat and marimastat, the human analogue of batimastat, revealed undesirable side effects which led to the development of more selective MMP inhibitors. Selective MMP inhibitors have been shown to affect colon cancer progression and metastasis in animal models as well. CT1746 (Celltech; a selective MMP-2, MMP-3 and MMP-9 inhibitor) reduced both primary tumor growth, tumor spread and metastasis of a human colon cancer cells (Co-3) in nude mice [82]. Drummond et al. [83] showed that administration of the MMP inhibitor BAY12-9566 (a selective MMP-2, MMP-3 and MMP-9 inhibitor) reduced primary tumor growth by 35% and distant metastasis by 50% in nude mice. Moreover, the sizes of metastases were smaller, indicating that the effect of the inhibitor is at the level of both cancer cell dissemination and tumor growth. The MMP inhibitor R-94138, which is selective for MMP-2 and MMP-9, reduced in vitro invasiveness of HT-29 and LS174T cells, two colorectal cancer cell lines that both express MMP-2 and MMP-9, and reduced the numbers of lymph node metastases in vivo [84]. KB-R7785, a selective inhibitor of MMP-1, MMP-3 and MMP-9, showed 90% inhibition of primary tumor growth of C-26 colon cancer cells in a transparent chamber model and strongly reduced the number and sizes of lung metastases [85]. MMI-166, a selective inhibitor of MMP-2 and MMP-9, reduced the number of mice that developed liver metastases from 80% to 20% after orthotopical implantation of fragments of tumors of colon cancer cells (TK-4) as well as growth of the primary tumor [86]. Administration of MMI-166 or BPHA, a selective inhibitor of MMP-2, MMP-9 and MT1-MMP, to nude mice after injection of C-1H colon cancer cells into the spleen resulted in reduction of liver metastases by 60% and 40%, respectively [87,88]. Simultaneous treatment of animals with MMI-166 and chemotherapy with mitomycin C or Irinotecan (CPT-11) synergistically reduced liver metastases of TK-4 and C-1H colon cancer cells, respectively [89,90]. It can be concluded from these studies that application of selective MMP inhibitors reduces colon cancer progression and metastasis. Similar effects of broad-range inhibitors and selective MMP inhibitors suggest a large contribution of gelatinases in colon cancer progression and metastasis. However, one should interpret such results with care since 'selective' MMP inhibitors applied at high doses can have a broad inhibitory profile. Finally, simultaneous treatment with MMP inhibitors and cytotoxic agents is synergistic in the efficacy of colorectal cancer treatment.

5. Regulation of MMP-2 and MMP-9 expression

The promotors of MMP genes are under control of growth factors, cytokines and tumor promotors via signal transduction pathways. Three mitogen-activated protein kinase (MAPK) pathways are known to regulate MMP-2 and MMP-9 expression, p38 kinase, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). The p38 kinases and JNKs are generally activated in response to inflammatory cytokines, osmotic stress and apoptotic signals. ERKs generally respond to cytokines, growth factors and phorbol esters. As a consequence, a group of protein kinases [MAPK kinase kinases (MAPKKKs)] phosphorylate MAPK kinases (MAPKK) which phosphorylate and activate MAPK. Active MAPKs translocate to the nucleus and activate a series of transcription factors that interact with transcription factor binding sites in MMP promotors (Fig. 1).

The MMP-2 gene has long been considered not to be sensitive to modulation due to a lack of well-characterized regulatory elements in the MMP-2 promotor region [91]. However, sequence analysis of the human MMP-2 promotor region revealed a number of *cis*-acting regulatory elements including binding sites for cAMP-response element-binding protein (CREB), p53, Ets-1, CCAAT/enhancer-binding protein (C/EBP), Sp1 consensus site (GC box), AP-1 binding site (TPA responsive element (TRE)) and an AP-2 binding site which may be involved in the regulation of MMP-2 expression [92]. One *cis*-acting enhancer element was designated r2 in man or RE-1 in rat and contains binding motifs for the transcription factors activating protein-2 (AP-2), p53 and Y-box transcription factor (YB-1; Fig. 2). Bian and Sun [93] showed that activation of the MMP-2 promotor was dependent on a p53-binding site. The transcription factors AP-2 and YB-1 were shown to synergistically enhance MMP-2 expression due to heteromeric AP-2-YB1 complex binding to the RE1 element [94]. Afterwards, it was found that AP-2/p53 and AP-2/YB-1 act together in complexes, whereas YB-1 competes with p53 for binding. The combination of AP-2, YB1 and p53 transcription factors resulted in a major increase in RE1 binding [95]. Furthermore, nm23-β in rat or nm23-H1 in man competes for the YB-1 site resulting in a dose-dependent reduction in MMP-2 expression [96]. Sp1 and Sp3 transcription factors, which both bind to Sp1 binding sites, are activators of the MMP-2 promotor and are synergistic in enhancing MMP-2 expression [92]. Sp1 activation was shown to be dependent on ERK activity via MKK1 and can be suppressed by nonsteroidal anti-inflammatory drugs (NSAIDs) [97]. Fibroblasts immortalized with SV40 large T-antigen (TAg) but not normal

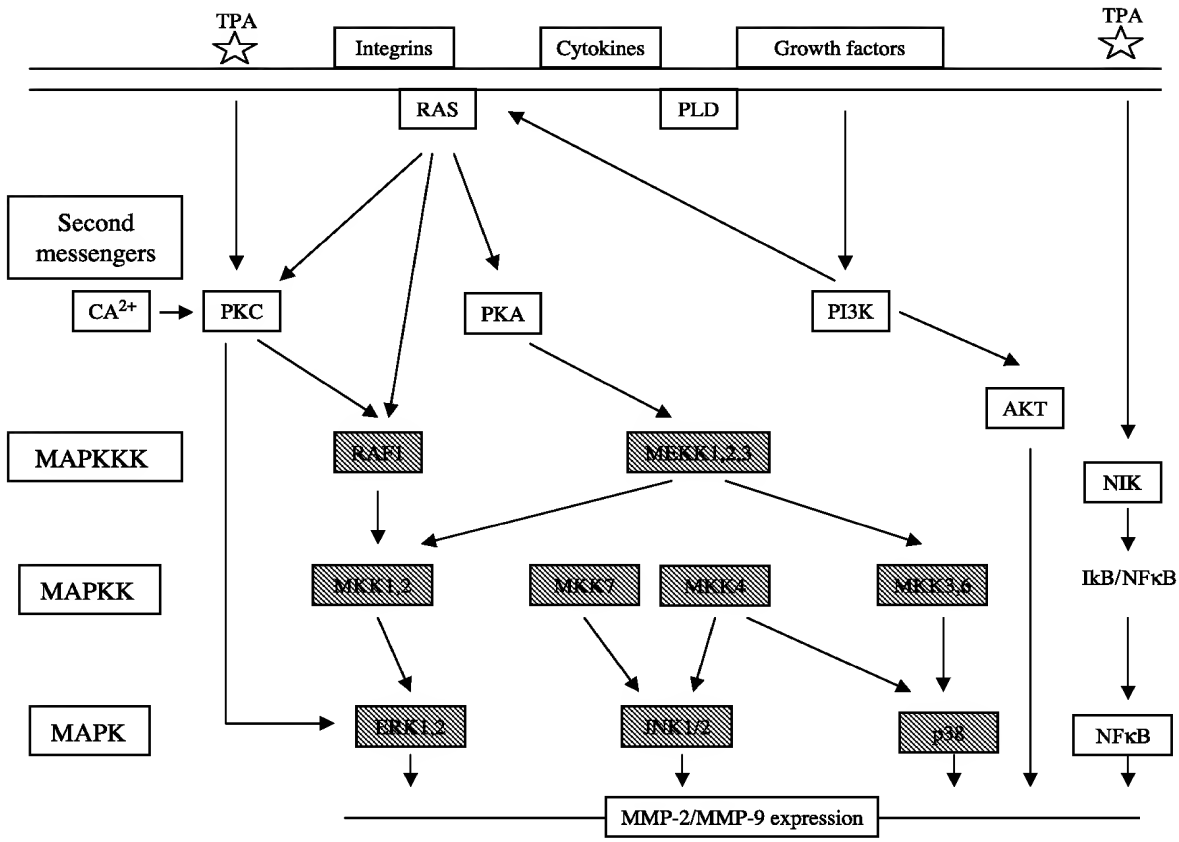


Fig. 1. General schematic representation of signalling pathways that regulate MMP-2 and MMP-9 expression. Components of the MAPK signalling pathway are depicted in grey. Extracellular signals like phorbol ester (TPA), integrins, growth factors and cytokines activate MAPK. Active MAPK can regulate MMP-2 and MMP-9 expression.

fibroblasts require c-jun to sustain expression of MMP-2 [98]. On the other hand, ATF3 suppressed MMP-2 promotor activity as an antagonist of p53 [99]. Active serine/threonine kinase (AKT) stimulates MMP-2 expression whereas activation of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) attenuates AKT activity and likely suppresses MMP-2 expression [100] (Fig. 3). Therefore, it can be concluded that MMP-2 expression is constitutive but MMP-2 promotor activity is also inducible. Regulation is dependent on the activation of

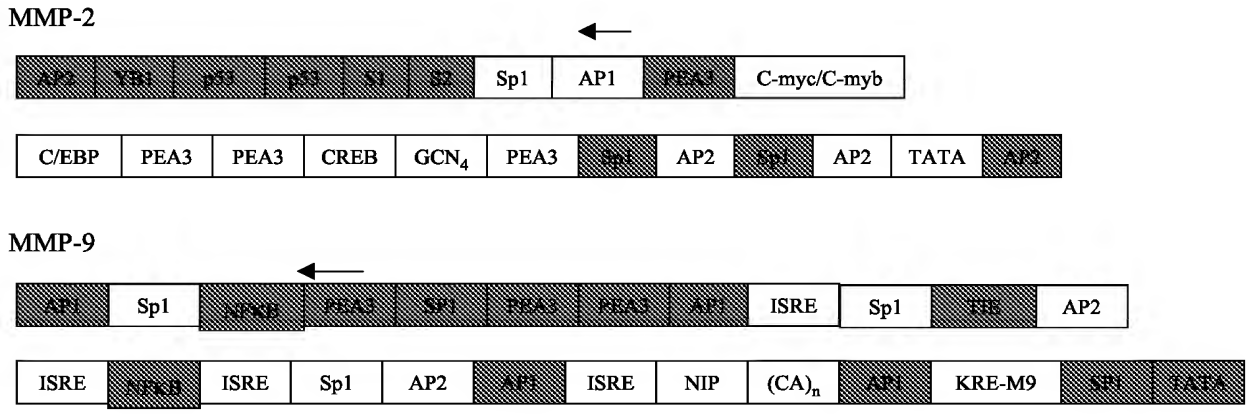


Fig. 2. Potential regulatory elements in the promotor regions of the MMP-2 and MMP-9 genes. The relative positions of the regulatory elements, indicated as boxes, are not drawn to scale. Regulatory elements with established physiological roles are depicted in red. Transcription-factor binding sites include AP1 (activator protein 1 sites), AP2 (activator protein 2 sites), c-myc/c-myb (proto-oncogene found in avian myeolocytomatosis virus), $(CA)_n$ (CA box), C/EBP (CCAAT/enhancer binding protein site), CREB (cyclic AMP-response element binding protein site), GCN_4 (yeast transcriptional activator), ISRE (interferon-stimulated regulatory element), KRE-M9 (keratinocyte differentiation factor-1 regulatory element), NFκB (nuclear factor-κB site), NIP (nuclear inhibition protein), p53 (p53 binding site), PEA3 (polyoma enhancer A binding protein-3 site), S1 and S2 (silencer sequence 1 and 2 site), Sp1 (Sp-1 binding/consensus site), TIE (transforming growth factor-β inhibitory element), and YB1 (Y-box transcription factor binding site).

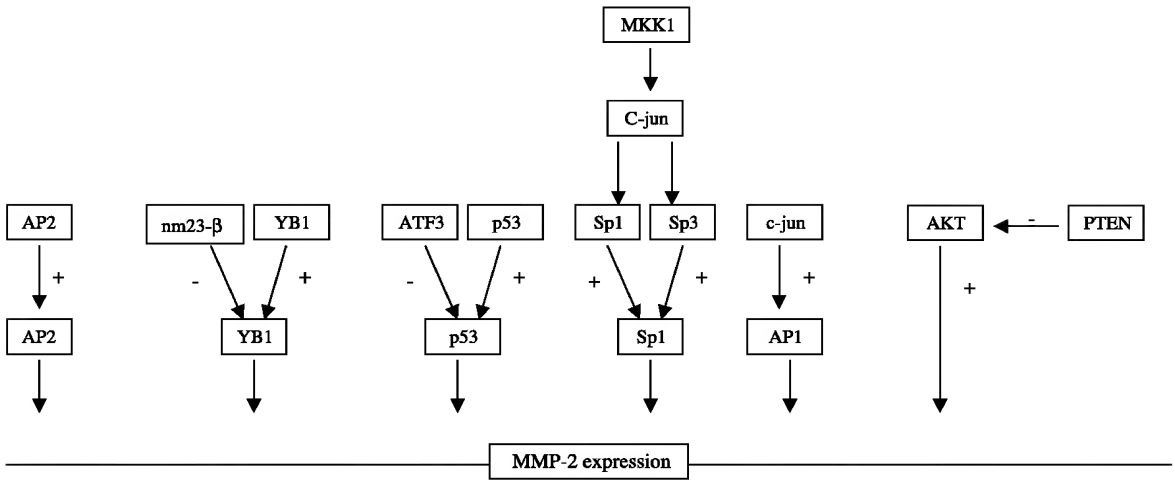


Fig. 3. Positive and negative regulators of MMP-2 expression. (+) Up-regulation of MMP-2 expression; (−) down-regulation of MMP-2 expression.

several transcription factors that can both up-regulate and down-regulate MMP-2 promotor activity.

The MMP-9 promotor has several transcription factor-binding motifs including AP-1 and AP-2 binding sites, nuclear factor κB (NFκB) consensus sites, stimulating protein 1 (SP1) sites and polyoma enhancer A binding protein-3 (PEA-3) sites, GT-boxes, interferon-stimulated regulatory element (ISRE), transforming growth factor-β inhibitory element (TIE), keratinocyte differentiation factor-1 regulatory element (KRE-M9), and nuclear inhibition protein (NIP) binding sites [101] (Fig. 2).

MMP-9 expression is regulated by binding of multiple factors to their response elements. NFκB up-regulates MMP-9 transcription via the tumor necrosis factor α (TNFα) signalling pathway due to a recognition sequence at −600 bp [102]. Interferons down-regulate MMP-9 expression by activation of interferon regulatory factor 1 (IRF1) and signal transducer and activator of transcription 1 (STAT1), which compete with binding of NFκB [103]. KiSS-1 diminishes MMP-9 expression by reducing NFκB binding to the promoter [104]. Active c-fos inhibits MMP-9 expression via the AP-1 site at −79 bp [105], whereas signals which activate JNK1 or active c-jun stimulates MMP-9 expression via AP-1 at −79 bp [102]. Thus, opposite effects on MMP-9 expression can be mediated via the same recognition site, depending on which binding proteins are activated. This was also found for ETS transcription factors. ETS-2 induces MMP-9 promotor activity whereas myocyte enhancer factor (MEF), an ETS transcription factor, reduces MMP-9 promotor activity probably due to competition for the same ETS site [106]. Stimulation of MMP-9 expression by phorbol ester was reported to be both p38-dependent [107] and AP-1 (−79 bp)-dependent and MMK6 was shown to be involved in AP-1-dependent MMP-9 promotor activity [108]. A hydroxymethylglutaryl coenzyme A reductase inhibitor which affects Ras signalling decreases MMP-9 but not MMP-2 expression in NIH 3T3 fibroblasts and Ras-transformed NIH 3T3 fibroblasts (Fig. 4). In addition, lovastatin also reduced AP-1- and NFκB-binding activities [109]. In conclusion, all three MAPKs, i.e., ERK, JNK and p38, can be involved in the regulation of MMP-9 transcription. Activation of transcription factors can either up-regulate or down-regulate MMP-2 and MMP-9 expression. This is dependent on which transcription factor is activated but can also be dependent on the cell type.

5.1. Extracellular signals involved in regulation of MMP-2 and MMP-9 expression

The intracellular signalling cascades that are involved in regulation of expression of MMP-2 and MMP-9 can be induced by extracellular signals such as growth factors, cytokines, mitogens, environmental stress and interactions with ECM components.

5.2. Effects of growth factors and cytokines on gelatinase expression by colon cancer cells

Transforming growth factor β (TGFβ) induces secretion of MMP-9 by colon cancer cells in a cell line-dependent

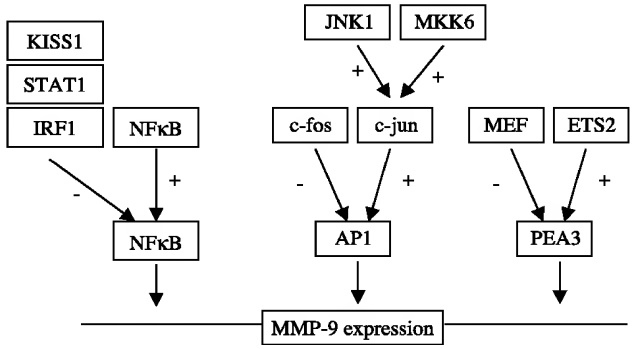


Fig. 4. Positive and negative regulators of MMP-9 expression. (+) Up-regulation of MMP-2 expression; (−) down-regulation of MMP-2 expression.

manner. Highly metastatic cells (LuM1) showed a strong response, whereas poorly metastatic cells (NM11) were not affected very much [110]. Furthermore, a regulatory role of TGFβ1 in invasion and metastasis-related processes has been illustrated by differences in matrigel invasiveness of two colon cancer cell lines. U9 and HD3 colon cancer cells expressed similar amounts of TGFβ1 and its receptor but responded differently to TGFβ1. The U9 cell line exhibited autocrine up-regulation of growth in vitro by TGFβ1, whereas HD3 cells showed an opposite response, an autocrine down-regulation of growth. U9 cells were 13-fold more invasive in matrigel than HD3 cells upon TGFβ1 stimulation [111]. This phenomenon strongly suggests that signalling events downstream of the TGFβ receptor are involved in MMP-9 secretion and that signalling events are cell line-dependent. Colon cancer cells (U9A) transfected with antisense TGFβ1, which expressed a third of the amounts of TGFβ1 mRNA and protein in comparison with parental cells, were severely growth-retarded in vivo and had threefold increase in lag time in anchorage-dependent colony formation in vitro. The parental cell line was 44-fold more invasive in matrigel than the transfected cell line [112]. Therefore, it appears that invasiveness of colon cancer cells can be modulated by TGFβ1. Keeping in mind that the liver is a major organ for colorectal cancer metastasis, it is interesting to note that significantly more active TGFβ1 is released in a coculture of human colon carcinoma cells and hepatocytes than in cultures of the cancer cells or hepatocytes alone [113]. These data indicate that cell–cell interactions are responsible for the release of active TGFβ1. Moreover, it has been shown that TGFβ1 is involved in stabilization of MMP-9 mRNA and MMP-2 proenzyme in prostate cancer cells [114]. Thus, TGFβ may induce invasion of colon cancer cells by induction of gelatinase activity.

Besides TGFβ, other growth factors and cytokines can regulate MMP-9 secretion by colon cancer cells. For example, secretion of MMP-9 by LuM1 cells is enhanced by IL-1β and TNFα [110]. However, TNFα can exert different effects on MMP-9 expression. TNFα up-regulates MMP-9 expression via NFκB and down-regulates MMP-9 expression via interferons (IFNs). In vitro administration of IFNα and IFNβ, but not IFNγ, down-regulates basic fibroblast growth factor (bFGF) expression in colon cancer cells [115]. bFGF is a known inducer of MMP-2 and MMP-9 expression and, therefore, down-regulation of bFGF may have an effect on MMP expression. Colon cancer tumor growth, tumor vascularization and expression of bFGF and MMP-9 mRNA and protein were similarly reduced in mice after treatment with IFNα [116]. Therefore, the effects of IFNα and IFNβ on MMP-2 and MMP-9 expression can be mediated via signalling pathways that down-regulate bFGF expression. IFNγ down-regulates MMP-9 expression directly via different signalling molecules [103] and inhibits MMP-9 expression independently of bFGF expression.

Hepatocyte growth factor (HGF) has been demonstrated to promote invasiveness of colon cancer cells (Caco-2) in matrigel with concomitant enhancement of protease production. Administration of an antibody against HGF inhibited both protease production and invasiveness of cancer cells. A synthetic general MMP inhibitor and neutralizing antibodies against MMPs and uPA significantly inhibited HGF-promoted cell invasion. Moreover, specific inhibitors of protein kinase C (PKC)α/β1 and phosphoinositide-3 (PI3) kinase decreased both HGF-induced cell invasion and protease expression. Therefore, PI3 kinase and PKC seem to be involved in HGF-induced invasiveness of these colon cancer cells at two levels, namely by induction of cell motility and overproduction of proteases [117]. Invasion in matrigel of L-10 colon cancer cells was also stimulated by HGF with concomitant expression of MMP-2. This process was inhibited by batimastat, TIMP-1 and TIMP-2 [118]. Matrigel invasion by colon cancer cells, which express HGF receptors and IL-4 receptors, was significantly increased by exogenous HGF. HGF stimulated production of MMP-1, -2, and -9 by these cell lines. These phenomena were antagonized by IL-4 and it was proposed that IL-4 is a potent inhibitor of HGF-induced cancer cell invasion [119]. ERK-MAPK was shown to be involved in HGF-mediated MMP-9 expression in keratinocytes. The duration of ERK activation was shown to be crucial for MMP-9 expression because only HGF and epidermal growth factor (EGF) induced sustained ERK activation and concomitant MMP-9 expression, whereas transient ERK activation by insulin-like growth factor (IGF) and keratinocyte growth factor (KGF) did not [120]. This signalling pathway may be similar in colon cancer cells since EGF was shown to induce MMP-9 secretion in colon cancer cells (LuM1) as well [110]. DLD-1 colon cancer cells overexpress the receptor tyrosine kinase (RTK) c-kit and its ligand stem cell factor (SCF), thereby establishing an autocrine c-kit mediated loop which regulates several cellular responses. One of the responses of exogenous SCF is up-regulation of MMP-9 expression, but not of MMP-2 expression, which is required for matrigel invasion [121]. These findings show that a number of growth factors and cytokines are involved in the regulation of gelatinase expression in colon cancer cells (Table 3).

5.3. Effects of growth factors and cytokines on gelatinase expression by non-cancer cells

Cancer cells can be stimulated to express MMP-2 and MMP-9 by different growth factors and cytokines but cancer cells can also secrete factors that induce gelatinase expression in non-cancer cells (Table 3). This is especially interesting since MMP-2 and MMP-9 expression in many colorectal tumors is maily found in stromal cells and this expression in stromal cells is also regulated by growth factors and cytokines. For example, TGFβ1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are pro-

Table 3
The effect of growth factors and cytokines on MMP-2 and MMP-9 expression and secretion in different cell types

Cell type	Stimulus	Effect on MMP-2	Effect on MMP-9
Colon cancer cell	TGFβ		Secretion ↑, secretion =
	IL-1β		Secretion ↑
	TNFα		Expression ↑, expression ↓
	IFNα and IFNβ (via bFGF ↓)	Expression ↓	Expression ↓
	IFNγ (bFGF independent)		Expression ↓
	HGF	Expression ↑	Expression ↑
	HGF- IL-4	Expression ↓	Expression ↓
	EGF		Secretion ↑
	SCF	Expression =	Expression ↑
Fibroblast	TNFα	Secretion =, Expression ↑	Secretion ↑, Expression ↑ + (PDGF,bFGF)
	IL-1β	Secretion =, Expression ↑	Secretion ↑
	IL-1α	Expression ↑	Expression ↑ + (PMA, oncostatin M, PDGF, TNFβ, bFGF)
	TGFβ	Expression ↑	Expression ↑ + (IL-1β, TNFα)
	IFNγ	Expression ↑	
Monocytes	TNFα	Secretion ↑	
	MCP-1	Expression ↑	
	TGFβ	Expression ↑	
	GM-CSF		Expression ↑
	IL-1β		Expression ↑ + (IL-1β, MCP-1, M-CSF, PMA, TGFβ, TNFα)
	TNFα		Expression ↑ + (IL-1β, MCP-1, M-CSF, PMA, TGFβ, TNFα)
	RANTES		Expression ↑, Secretion ↑
	MIP-1α		Expression ↑, Secretion ↑
	IL-4		Expression ↓
	IL-10	Expression =	Expression ↓
Macrophages	Cytokines in general	Expression =	
	GM-CSF		Expression ↑
	PMA		Expression ↑
	IL-1β		Expression ↑
	IFNγ		Expression ↓+ (IL-4)
	IL-4		Expression ↓
	IL-10		Expression ↓
T-lymphocytes	IL-2	Expression ↑	Expression ↑
	IL-1, MIP-1α, MIP-1β, PMA, RANTES and TNFα		Expression ↑
	IL-4		Expression ↓
	IL-10		Expression ↓
CD34+	CSF, G-CSF, GM-CSF, M-CSF, SDF-1, IL-3, IL-6, IL-8, TNFα	Expression ↑	Expression ↑
Endothelial cells	HGF, VEGF	Expression ↑	
	TNFα		Expression ↑
	PMA		Expression ↑ + (IL-1α, TNFα)

(=) No effect; (↑) inducing effect; (↓) repressive effect; (+) synergizes with; (−) antagonizes with.

duced by malignant colon cancer cells and may induce cell contact-independent up-regulation of MMP-2 and MMP-9 secretion by monocytes [72]. Furthermore, colon cancer cell lines have been reported to secrete platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), TNFα, TGFα, IGF-2, IL-1 and IL-10 [59, 122]. Furthermore, expression of IL-8 by colon cancer cells is associated with their potential to metastasize [123]. This implies that secretion of growth factors and cytokines by colon cancer cells can stimulate gelatinase expression in host cells.

TNFα and IL-1β stimulated secretion of MMP-9, but not of MMP-2, TIMP-1 or TIMP-2 in cultured human fibroblasts in the study of Wong et al. [124], but induction of MMP-2 expression by IFNγ, IL-1α, IL-1β, TGFβ and TNFα has also been reported. Furthermore, MMP-9 in fibroblasts is up-regulated by IL1α alone or synergistically with phorbol 12-myristate 13-acetate (PMA), oncostatin M, PDGF, TNFβ. MMP-9 in fibroblasts is up-regulated by TGFβ alone and in synergism with both IL-1β and TNFα. Finally, IL-1α and TNFα interact synergistically on MMP9 expression with either PDGF or bFGF (Table 3; reviewed in Ref. [125]).

MMP-2 secretion in monocytes is induced by TNFα [126] and MMP-2 expression in monocytes is up-regulated by monocyte chemoattractant protein-1 (MCP-1) and TGFβ [127,128], whereas MMP-9 is up-regulated by GM-CSF. MMP-9 expression is also up-regulated by IL-1β and TNFα, which act synergistically with IL-1β, MCP-1, M-CSF, PMA, TGFβ and TNFα. Furthermore, MCP-2 [129] and macrophage inflammatory protein 1α (MIP-1α) induce MMP-9 gene expression and secretion in monocytic cell lines and peripheral blood monocytes [130]. IL-4 down-regulates the production of monocyte MMP-9 via prostaglandin E_2 (PGE_2) [131] whereas IL-10 is a suppressor of monocyte MMP-9 expression but does not affect MMP-2 expression [132] (Table 3). In macrophages, MMP-2 expression is not induced by cytokines whereas MMP-9 is up-regulated only by GM-CSF, PMA and IL-1β. On the other hand, IFNγ, IL-4 and IL-10 down-regulate MMP-9 expression and IFNγ and IL-4 synergistically abolish MMP-9 expression in macrophages almost completely (Table 3).

MMP-2 expression in T-lymphocytes is up-regulated by IL-2 whereas IL-1, IL-2, MIP-1α, MIP-1β, PMA, RANTES

and TNFα can up-regulate MMP-9 (Table 3). MMP-2 and MMP-9 are induced in steady-state bone marrow progenitor cells (CD34+) by CSF, granulocyte-CSF (G-CSF), GM-CSF, macrophage-CSF (M-CSF), stromal cell-derived factor-1 (SDF-1), IL-3, IL-6, IL-8 and TNFα with concomitant increased matrigel invasiveness (Table 3) [133,134]. MMP-2 expression in endothelial cells is up-regulated by HGF and VEGF whereas MMP-9 is up-regulated by TNFα and PMA alone and by PMA in synergy with IL1α or TNFα (Table 3).

In conclusion, growth factors and cytokines are strongly involved in regulation of MMP-2 and MMP-9 expression in cancer cells and stromal cells. The effects of growth factors and cytokines are different for MMP-2 and MMP-9 and, more importantly, are cell-type dependent. Various combinations of cytokines that are present in inflammatory sites, as well as their balance in the different stages of inflammation may provide signals necessary for regulating MMP expression. Colon cancer cells can also contribute to a local increase in growth factors and cytokines and thereby stimulate gelatinase production and secretion by stromal cells.

6. ECM components

Cell–ECM interactions provide cells with information on their environment. Loss of cell–ECM interactions, changes in expression of adhesion molecules or changes in the composition of the ECM can have profound effects on the behavior of cancer cells and stromal cells. Modified Arg-Gly-Asp (RGD) peptides inhibit endothelial cell adhesion and growth in vitro whereas a single dose of modified RGD peptides results in decreased tumor angiogenesis in vivo [135]. Furthermore, administration of both a modified RGD sequence and metastatic colon cancer cells (26-L5) into the portal vein of mice resulted in a marked suppression of the numbers of liver metastases in a dose-dependent manner. The modified RGD sequence inhibited degradation of gelatin by MMPs produced by colon cancer cells (26-L5) [136]. These data imply that integrins are also involved in regulation of MMP expression. Furthermore, αvβ6 integrin expression in colon cancer cells (SW480) was shown to correlate with induction of MMP-9 expression and concomitant elevated matrigel invasion [137]. An ERK2-binding site was identified on the β6 integrin subunit and interactions between β6 and ERK2 were shown to be responsible for induction of MMP-9 expression in WiDr and HT-29 colon cancer cells [138]. It was shown that MMP-9 expression in colon cancer cells is mediated by the unique C-terminal cytoplasmic tail of the β6 integrin subunit that is linked to the PKC signalling pathway [139]. Therefore, MMP-9 expression can be up-regulated via the unique cytoplasmic tail of β6 that can bind ERK2 and then involves PKC-mediated signalling. Furthermore, it has been shown that αvβ6 binds to and activates latent TGFβ1 and TGFβ3 via interactions with their pro-peptide, the latency-associated peptide (LAP) [140]. As described above, TGFβ is an inducer of MMP-9 expression. β6-Transfected colon cancer cells revealed that LAP, which contains a RGD sequence, is a high-affinity ligand for αvβ6 and that the interactions of LAP with αvβ6 induce phosphorylation of downstream components of integrin-signalling complexes which can also be involved in regulation of MMP-9 expression [141]. This defines a novel paradigm of integrin-mediated signalling in colon cancer because non-ECM components act on an ECM-binding site.

Simultaneous administration of antibodies against the β4 integrin subunit and colon cancer cells (LoVo C5) stimulated metastasis in vivo, which is in line with the fact that both invasiveness in matrigel and MMP-2 expression are induced by antibodies against β4 [142]. PI3K is assumed to be involved in the signalling cascade. Interestingly, α6β4, which is the only known dimer that involves β4, is a laminin receptor. This may explain how laminin 2-binding of cells can induce MMP-2 expression. Apparently, laminin binding induces signal transduction leading to MMP-2 expression probably mediated also via phospholipase D (PLD) and its product phosphatidic acid [143]. Fibronectin binding to the fibronectin receptor α5β1 induced increased expression of MMP-2 in melanoma cells [144] and MMP-9 secretion by ovarian cancer cells. These effects are dependent on dual activation of both the MAPK/ERK kinase (MEK1) and the PI3K signalling pathways [145]. Furthermore, α3β1, in association with tetraspanin, has been implicated in PI3K-dependent MMP-2 production and matrigel invasion of MDA-MB-231 cells [146]. It has been shown that divalent ligation of β1 integrins stimulated expression of pro-MMP-2 in ovarian cancer cells [147]. Therefore, it can be concluded that interaction of cancer cells with ECM components via integrins can modulate gelatinase expression and the invasive capacity of cancer cells.

Not only cancer cells but also stromal cells can modulate their gelatinase expression in response to integrin/integrin substrate interactions. For example, colon cancer fibroblast cell–cell contacts up-regulate MMP-9 in fibroblasts and this process was inhibited completely by an antibody against the integrin subunit β1 and partly by the integrin subunits α5 and αv [69]. MMP-9 expression is up-regulated in macrophages grown on collagen type IV and fibronectin whereas laminin up-regulates both uPA and MMP-9. Up-regulation of uPA but not MMP-9 upon interactions with laminin is mediated by α6β1 integrin [148] whereas interactions of α5β1 with fibronectin are suggested to induce MMP-9 gene expression in differentiating macrophages [149]. T-cell adhesion via α4β1 to CS-1 peptide of fibronectin induced expression of MMP-2 and MMP-9. Interactions of α4β1 with vascular cell adhesion molecule 1 (VCAM-1), the second major α4β1 ligand, induced expression of MMP-2 but failed to induce MMP-9 expression. Apparently, adhesion of T-cells via α4β1 to different ligands triggers intracellular events leading to distinct patterns of MMP expression [150]. MMP-9 is expressed constitutively in resting T-cells, whereas MMP-2 expression is induced and

MMP-9 expression is increased upon T-cell activation. Migration of resting T-cells in matrigel is mediated by MMP-9 whereas activated T-cells in principle can use both gelatinases for matrigel invasion [65]. Fibronectin up-regulates MMP-9 and induces MMP-2 and MT1-MMP expression whereas TIMP-2 expression is down-regulated in T-cell lines. Blocking antibodies against the integrin subunits α4, α5 and αv strongly reduced fibronectin-induced MMP-2 and MMP-9 production in T-cells [151].

Degradation of the ECM can also reveal cryptic sites, which in turn can affect MMP expression. For example, proteolytic degradation of laminin-1 by elastase generates laminin-1 fragments which stimulate MMP-9 expression in macrophages via phosphorylation of MAPK (ERK1/2). In contrast, laminin fragments generated by MMP-2, MMP-7 or plasmin did not exert this effect [152].

In conclusion, both animal studies and in vitro studies have shown the involvement of interactions between colon cancer cells as well as stromal cells and ECM in the regulation of MMP-2 and MMP-9 expression. This is dependent on the type of integrin expressed and the type of ECM components that are encountered. Interestingly, degradation of ECM can also reveal cryptic binding sites, which in turn affect MMP expression.

6.1. Activation of MMP-2 and MMP-9

MMP-2 and MMP-9, like most proteolytic enzymes, are expressed and secreted in a biologically inactive proform. MMP-2 and MMP-9 can be activated non-enzymatically by aminophenyl mercuric acid (APMA). However, activation in vivo (usually) occurs by proteolytic removal of the pro-sequence by other proteases (Fig. 5). The mechanism of MMP-2 activation has been largely elucidated. Pro-MMP-2 activation occurs in a ternary complex with TIMP-2 and MT1-MMP. Complex formation between MT1-MMP and TIMP-2 seems to be an initial step, which is followed by binding to pro-MMP-2. Then, pro-MMP-2 is activated by a TIMP-2-free active MT1-MMP molecule [153]. In this way, TIMP-2 levels are crucial for pro-MMP-2 activation since excess of TIMP-2 lowers the free MT1-MMP levels and low levels of TIMP-2 fail to localize pro-MMP-2 to the cell surface [154]. The integrin αvβ3 is involved in docking of MMP-2 to the plasma membrane, but it is still a matter of debate whether it is actively involved in pro-MMP-2 activation [155, 156]. All MT-MMPs, except MT4-MMP, are able to activate MMP-2 [157–159], whereas MMP-9 is not activated by any MT-MMP. Trypsin-2 can activate pro-MMP-9 and partially activate pro-MMP-2 [160]. Down-regulation of trypsinogen 2 prevents activation of pro-MMP-9 in colon cancer cells [161]. In ovarian cancer, high levels of trypsin have been associated with activation of pro-MMP-9 but not of pro-MMP-2 [162]. Both pro-MMP-9 and pro-MMP-2 can be activated by stromelysin-1 (MMP-3) [163, 164], collagenase-1 (MMP-1) [164, 165], matrilysin-1 (MMP-7) [164, 165] and the uPA–plasmin cascade [166–168]. Furthermore, pro-MMP-9 can be activated by MMP-2 [169], stromelysin-2 (MMP-10) [170], collagenase-2 (MMP-13) [171] and matrilysin-2 (MMP-26) [172]. In fact, MMP-2 and MMP-9 can be activated by a large series of active proteases. However, it has to be realized that these proteases have to be activated by other proteases and, thus, it is likely that proteolytic cascades are needed to activate MMP-2 and MMP-9. Here, we focus on direct activators of MMP-2 and MMP-9 (Fig. 5) without discussing complete proteolytic cascades.

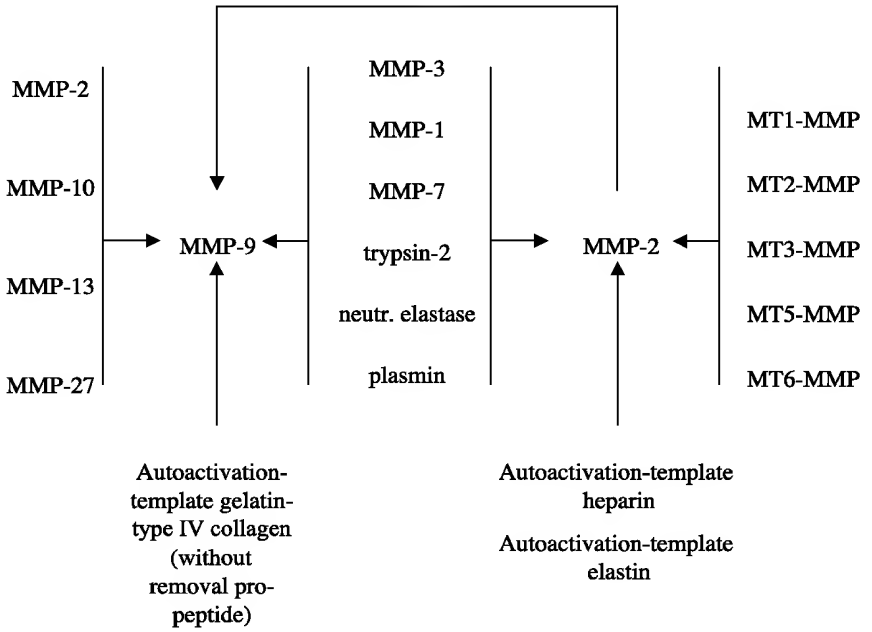


Fig. 5. All known direct activators of MMP-2 and MMP-9. Although most activators are proteases that have to be activated by other proteases, i.e., activation via proteolytic cascades, we focused on the direct activators off MMP-2 and MMP-9 without discussing their activators.

Gelatinases are unique in the MMP family in that they contain three fibronectin type II (FN-II)-like domains. FN-II domains of pro-MMP-2 and pro-MMP-9 are mainly involved in binding to ECM. This binding can be relevant for the fate of gelatinases. Extracellular autoproteolytic activation of MMP-2 has been described by Crabbe et al. [173] and may involve binding of MMP-2 to ECM components as was demonstrated in the process of auto-activation of MMP-2 that is accelerated by heparin [173] and elastin [174] by a template mechanism. In analogy with template activation, binding of MMP-9 to gelatin or collagen type IV has been proposed to be a novel activation mechanism for MMP-9 [175]. After ligand or substrate binding, MMP-9 becomes proteolytically active without removal of the pro-sequence (Fig. 5).

In conclusion, activation of pro-MMP-2 and pro-MMP-9 occurs by a number of activated proteases including other MMPs, the uPA–plasmin cascade and trypsin. Binding of gelatinases to ECM components can lead to activation of these enzymes due to accelerated auto-activation or activation without removal of the pro-sequence.

7. MMPs have other substrates than ECM components

Traditionally, MMPs are believed to be mainly involved in degradation of ECM, thereby facilitating cell migration. However, many non-ECM proteins are substrates for MMP-2 and MMP-9 as well. These include proteins involved in growth, immune response and angiogenesis independent of ECM destruction. The discovery of such mechanisms of gelatinases has put the role of gelatinases in cancer progression into a different perspective.

7.1. Growth factors and cytokines

Growth factors can be sequestered in the ECM and can be liberated upon matrix remodelling. The majority of growth factors are present in an inactive pro-form, such as EGF, HB-EGF, TGFα, TNFα, c-kit ligand and CSF-1, or are bound, and thereby inactive, to binding proteins, such as IGF-1, IGF-2, TGFβ and FGF-2 [176]. MMPs can liberate inactive growth factors from the ECM and evidence is accumulating that MMPs can activate liberated growth factors or degrade binding proteins, which results in an increase in bioactive growth factors. For example, IGF bioactivity is controlled by several high-affinity binding proteins (IGFBPs). Excess of IGFBPs results in little free IGF. MMP-2 and MMP-9 can modulate IGF bioactivity by degradation of IGFBPs [176]. MMP-9 has been found to be localized at the cell surface in complex with CD44 and the complex can activate TGFβ in normal keratinocytes [177]. Pro-TNFα is activated by MMP-9, although at a lower rate than by other MMPs [178]. Synthetic MMP inhibitors and TIMP-2, but not TIMP-1, inhibit shedding of TNFα receptors from human colon cancer cell lines (Colo 205 and SW626) [179].

Another mechanism involving growth factors is reported by Levi et al. [180] who show that MMP-2, but not MMP-9, is involved in shedding of FGFR1. Shedding resulted in the release of an active ectodomain which can still bind FGF. Since FGF is an inducer of MMP-2 (see above), it may cause a feedback mechanism that can down-regulate cellular responses to FGF [180].

7.2. Immune responses

MMPs are expressed in white blood cells and participate in and promote inflammatory processes, but they may also blunt such processes. As discussed above, gelatinases are involved in the migration of many types of immune cells. In this way, immune cells can migrate towards cancer cells, which is a prerequisite for the recognition and killing of cancer cells. Gelatinase-dependent migration of immune cells is a direct link of gelatinases to the host defence system but, indirectly, gelatinases are also involved in host defence. For example, chemokines provide directional cues for leukocyte migration and activation that are essential for normal leukocyte trafficking and for host responses during processes such as inflammation, infection, and cancer. However, cancer cells have developed many ways to escape the immune system. Recent data show that MMP-2 and MMP-9 are involved in various processes that dampen the immune response. MCP3 was identified as a physiological substrate of MMP-2. Cleaved MCP3 can bind to CC-chemokine receptors 1, 2, and 3, but, unlike intact MCP-3, no longer induces intracellular calcium fluxes or promotes chemotaxis, but acts as a general chemokine antagonist that dampens inflammation instead [181]. Growth-related oncogene α (GRO-α), connective tissue activating peptide-III (CTAP-III) and platelet factor-4 (PF-4) are degraded into multiple fragments by MMP-9, thereby also dampening the immune response [60]. Another immune regulatory link was found by Sheu et al. [182] who showed shedding of IL-2Rα by MMP-9 activity. IL-2Rα–IL-2 interactions on T-cells initiate and propagate the immune response against cancer cells and receptor shedding dampens this response [182]. Active TGFβ is an important inhibitor of T-cell responses to tumors [183]. As mentioned above, MMP-9 is an activator of TGFβ and thus can contribute to inhibition of the T-cell response. Resistance of cancer cells to cytotoxic natural killer cells is increased when intercellular cell adhesion molecule 1 (ICAM-1) is shed from HL-60 cancer cells due to MMP-9 activity [184]. Furthermore, ICAM-1, CD44 and collagen type IV [185] are cell surface docking molecules for MMP-9 [186]. Therefore, localization of MMP-9 at the cell surface due to ICAM-1 and subsequent activation of MMP-9 can result in ICAM-1 shedding, thus increasing resistance of cancer cells to killing by immune cells.

On the other hand, pro-IL-1β can be activated by MMP-9 and to a lesser extent by MMP-2. It is noteworthy to recall

that IL-1β is a classical inducer of MMP expression, including MMP-2 and MMP-9, thus creating a positive feedback loop [187]. IL-8, also known as neutrophil chemoattractant CXCL8, is cleaved by MMP-9, resulting in a 10-fold increase in activity of IL-8. IL-8 induces the release of neutrophil MMP-9 thereby also generating a positive feedback loop. A similar mechanism was demonstrated for neutrophil MMP-9 and endothelin-1 (ET-1). The processing of ET-1 by MMP-9 generates ET-1 that induces the release of MMP-9 from neutrophils [178]. This suggests that MMPs are both effectors in migration of immune cells and regulators of the inflammatory response. Through its tightly regulated expression and its potentiating and activating effect on cytokines, MMP-9 acts more as a tuner and amplifier of immune functions than MMP-2. Thus, specific inhibition of MMP-9 may result in direct anti-inflammatory effects through inhibition of migration of leukocytes by blocking cleavage of matrix components and modification of chemokines.

7.3. Angiogenesis

Induction and formation of blood vessels is crucial for tumor progression. Angiogenesis is dependent on the capacity of endothelial cells to proliferate and migrate. Tumor growth is inhibited in MMP-2 knock-out mice due to impaired angiogenesis [188] and MMP-9 is involved in the angiogenic switch during carcinogenesis [189]. MMP-2 activity is involved in invasion of endothelial cells whereas MMP-9 may be involved as well in the generation of bioactive molecules that inhibit angiogenesis. For example, TNFα enhances angiostatin production by induction of uPA or tPA activity and the release of MMP-9 by prostate cancer cells. These studies support a model in which the antiangiogenic effects of TNFα on tumor microvasculature are mediated by generation of angiostatin by MMP-9 [190]. These results are in line with findings that MMP-9-transfected CT26 colon cancer cells, and not MMP-9-negative parental colon cancer cells, induce increased angiostatin serum levels. As a consequence, tumors of MMP-9-transfected cancer cells are smaller and less well-vascularized [191]. Furthermore, in vitro studies have shown that MMP-9, but not MMP-2, generates endostatin, a C-terminal fragment of collagen type XVIII [192]. Both angiostatin and endostatin are potent inhibitors of endothelial cell proliferation. In addition, endostatin inhibits endothelial cell invasion by prevention of pro-MMP-2 activation and activity [193]. MMP-2 and the integrin αvβ3 are functionally associated on the surface of angiogenic blood vessels. The C-terminal hemopexin-like domain of MMP-2 (PEX), which has no catalytic function, prevents interactions of active MMP-2 with αvβ3 on the cell surface of endothelial cells and inhibits angiogenesis in chick chorioallantoic membrane (CAM) models [194]. Organic molecules selected for binding to αvβ3 and concomitant inhibition of interactions with MMP-2 reduced angiogenesis in CAM models independently of integrin binding and MMP-2 activity [156], suggesting that only localized MMP-2 activity plays a role in angiogenesis. This suggests a dual role of MMP-2 and MMP-9 in tumor progression. Localized MMP-2 activity on endothelial cells plays a direct role in angiogenesis as it is involved in endothelial cell migration. MMP-9 is indirectly involved in angiogenesis. MMP-9 can act pro-tumorigenic as it plays a role in the angiogenic switch by modulation of VEGF mobilization, as well as anti-tumorigenic as it generates anti-angiogenic factors. In conclusion, gelatinases can be involved in cancer progression independent of matrix degradation. Gelatinases can modulate growth factor bioavailability, promote as well as dampen the immune response, and influence angiogenesis both positively and negatively.

8. Conclusions

A growing body of clinical and experimental evidence demonstrates that MMP-2 and MMP-9 play a role in colorectal cancer progression and metastasis. In the last decade, development of new techniques, cloning of TIMP genes and the synthesis of broad-range and selective MMP inhibitors have greatly improved our understanding of physiological and pathological roles of MMP-2 and MMP-9.

Early clinical evidence of the involvement of MMP-2 and MMP-9 in cancer progression was mainly based on correlations between colorectal cancer progression and MMP-2 and/or MMP-9 mRNA or protein expression. Later, it became evident that not absolute levels of MMPs but rather their active fraction is relevant. More importantly, the (im)balance between pro-enzyme activation and inhibition by TIMPs is being recognized as an important factor for colorectal tumor progression. Furthermore, in the case of TIMP-1 and TIMP-2, not only an inhibitory effect but also a growth-promoting effect has been observed in tumors. TIMPs may be inhibitors of cancer progression by inhibiting particular MMPs but may also promote cancer progression in a MMP-independent manner. It is still unclear how TIMP-1 and TIMP-2 are exactly involved in cancer progression.

Increased expression and proenzyme activation of MMP-2 and MMP-9 have made them attractive candidates for therapeutic intervention. Application of synthetic MMP inhibitors in preclinical studies has been shown in general to reduce cancer progression and metastasis. However, application of broad-range and selective synthetic MMP inhibitors in treatment of different types of cancer in clinical trials has shown only little clinical efficacy, no efficacy at all or even did worse than placebo treatment [195]. However, these trials were all performed on patients with advanced stages of cancer. Animal experiments showed that MMP inhibitors can be effective but only when they are administered in early stages of tumor

development. Therefore, MMP inhibitors are assumed to be useful as additive therapy for the prevention of cancer progression in early stage tumors or development of undetected micrometastases after surgery. However, the involvement of immune cells in tumor progression has been an issue of debate. Increased numbers of macrophages have been correlated with decreased metastatic potential. On the other hand, macrophage MMP-9 is proposed as pro-tumorigenic as it is used for matrix degradation necessary for tumor infiltration of surrounding tissues. Furthermore, immune cells are involved in the first line of defence against disseminating cancer cells. A wide range of immune cells express MMP-9, MMP-2 or both. The physiological role of MMP-2 and MMP-9 is most probably related to migration of immune cells towards and into inflamed tissue sites. In this process, gelatinases are postulated to be important for the host immune defence. In advanced tumors, in which the efficacy of the immune system is often down-regulated, immune cells may be a ready source of gelatinases to promote tumor progression.

A second physiological role of gelatinases in relation with the immune response is the activation and inactivation of cytokines that can either stimulate or dampen the immune response. When MMP inhibitors are used in early stages of cancer or to prevent the development of undetected micrometastases, one should keep in mind that the host defence also needs the same gelatinases. This means that when the host defence is still capable of clearing cancer cells, adverse effects of treatment with MMP inhibitors are a possibility.

MMP-9 is the gelatinase that is most involved in the immune system, but it has also been linked with the generation of antiangiogenic molecules. This raises the question which specific MMPs have to be blocked and whether inhibition of other proteases in combination with MMP inhibition will have stronger effects since metastatic disease is often delayed but not completely abolished in animal models by treatment with MMP inhibitors.

The rapidly expanding field of MMP research has shown that MMPs are not only ECM-degrading proteases. MMP-2 and MMP-9 have other functions as well. They are involved in growth factor release, growth factor activation, immune surveillance and angiogenesis, have pro-angiogenic and anti-angiogenic properties, generate bioactive matrix components and uncover cryptic binding sites in matrix molecules. In animal models, the final outcome of all these functions is tumor promotion, but some evidence has been presented that gelatinases can have a protective function against tumor development as well. The latter aspect of MMP-2 and MMP-9 may be the reason why most clinical trials with MMP inhibitors have been disappointing so far. Although many novel functions and mechanisms of action of MMP-2 and MMP-9 are being revealed, there are striking differences in the substrates of MMP-2 and MMP-9. In other words, although MMP-2 and MMP-9 are almost identical proteinases, their contribution to biological or pathological processes can be very different and it is unclear which of the two, MMP-2 or MMP-9, is most important in tumor progression and metastasis. Although MMP-2 and MMP-9 are structurally very similar, it is surprising that the substrate recognition profiles of MMP-2 and MMP-9 are strikingly different [196,197]. It has been speculated that more potential MMP substrates could be identified and these may differ in affinity for MMP-2 and MMP-9. In the development of anti-cancer therapies focussed on MMP-2 and MMP-9, these functions, and functions that are yet unknown, have to be considered in order to have a rationale for the development of therapy against colorectal cancer and metastasis.

References

[1] I. Taylor, Br. J. Surg. 83 (1996) 456–460.
[2] B.P. Himelstein, R. Canete-Soler, E.J. Bernhard, D.W. Dilks, R.J. Muschel, Invasion Metastasis 14 (1994) 246–258.
[3] W.C. Parks, R.P. Mecham, Matrix Metalloproteinases, Academic Press, San Diego, 1998.
[4] H. Nagase, J.F. Woessner Jr., J. Biol. Chem. 274 (1999) 21491–21494.
[5] L.J. McCawley, L.M. Matrisian, Mol. Med. Today 6 (2000) 149–156.
[6] G. Fassina, N. Ferrari, C. Brigati, R. Benelli, L. Santi, D.M. Noonan, A. Albini, Clin. Exp. Metastasis 18 (2000) 111–120.
[7] F. Mannello, G. Gazzanelli, Apoptosis 6 (2001) 479–482.
[8] C. Pyke, E. Ralfkiaer, K. Tryggvason, K. Dano, Am. J. Pathol. 142 (1993) 359–365.
[9] Z.S. Zeng, J.G. Guillem, Br. J. Cancer 74 (1996) 1161–1167.
[10] A.T. Levy, V. Cioce, M.E. Sobel, S. Garbisa, W.F. Grigioni, L.A. Liotta, W.G. Stetler-Stevenson, Cancer Res. 51 (1991) 439–444.
[11] C.C. Chan, M. Menges, H.D. Orzechowski, N. Orendain, G. Pistorius, G. Feifel, M. Zeitz, A. Stallmach, Int. J. Colorectal Dis. 16 (2001) 133–140.
[12] H.M. Collins, T.M. Morris, S.A. Watson, Br. J. Cancer 84 (2001) 1664–1670.
[13] D.L. Ornstein, K.H. Cohn, Dig. Dis. Sci. 47 (2002) 1821–1830.
[14] M.J. Heslin, J. Yan, M.R. Johnson, H. Weiss, R.B. Diasio, M.M. Urist, Ann. Surg. 233 (2001) 786–792.
[15] H. Masuda, H. Aoki, Dis. Colon Rectum 42 (1999) 393–397.
[16] H. Koumura, Y. Sugiyama, K. Kunieda, S. Saji, To Gan, Kagaku Ryoho 24 (Suppl. 2) (1997) 324–331.
[17] Z.S. Zeng, J.G. Guillem, Br. J. Cancer 72 (1995) 575–582.
[18] E. Roeb, C.G. Dietrich, R. Winograd, M. Arndt, B. Breuer, J. Fass, V. Schumpelick, S. Matern, Cancer 92 (2001) 2680–2691.
[19] Z. Zeng, Y. Sun, W. Shu, J.G. Guillem, Dis. Colon Rectum 44 (2001) 1290–1296.
[20] D.G. Powe, J.L. Brough, G.I. Carter, E.M. Bailey, W.G. Stetler-Stevenson, D.R. Turner, R.E. Hewitt, Br. J. Cancer 75 (1997) 1678–1683.
[21] K.J. Newell, J.P. Witty, W.H. Rodgers, L.M. Matrisian, Mol. Carcinog. 10 (1994) 199–206.
[22] Z.S. Zeng, A.M. Cohen, Z.F. Zhang, W. Stetler-Stevenson, J.G. Guillem, Clin. Cancer Res. 1 (1995) 899–906.
[23] Y. Ogata, K. Miura, A. Ohkita, H. Nagase, K. Shirouzu, Kurume Med. J. 48 (2001) 211–218.
[24] S.J. Urbanski, D.R. Edwards, N. Hershfield, S.A. Huchcroft, E. Shaffer, L. Sutherland, A.E. Kossakowska, Diagn. Mol. Pathol. 2 (1993) 81–89.
[25] M. Murashige, M. Miyahara, N. Shiraishi, T. Saito, K. Kohno, M. Kobayashi, Jpn. J. Clin. Oncol. 26 (1996) 303–309.

[26] K. Brew, D. Dinakarpandian, H. Nagase, Biochim. Biophys. Acta 1477 (2000) 267–283.
[27] T. Tomita, K. Iwata, Dis. Colon Rectum 39 (1996) 1255–1264.
[28] M.R. Emmert-Buck, M.J. Roth, Z. Zhuang, E. Campo, J. Rozhin, B.F. Sloane, L.A. Liotta, W.G. Stetler-Stevenson, Am. J. Pathol. 145 (1994) 1285–1290.
[29] T.S. Kim, Y.B. Kim, J. Korean Med. Sci. 14 (1999) 263–270.
[30] E.A. Baker, D.J. Leaper, Eur. J. Surg. Oncol. 28 (2002) 24–29.
[31] Z.S. Zeng, A.M. Cohen, J.G. Guillem, Carcinogenesis 20 (1999) 749–755.
[32] M. Jeziorska, N.Y. Haboubi, P.F. Schofield, Y. Ogata, H. Nagase, D.E. Woolley, Int. J. Colorectal Dis. 9 (1994) 141–148.
[33] S. Takeha, Y. Fujiyama, T. Bamba, T. Sorsa, H. Nagura, H. Ohtani, Jpn. J. Cancer Res. 88 (1997) 72–81.
[34] P. Ring, K. Johansson, M. Hoyhtya, K. Rubin, G. Lindmark, Br. J. Cancer 76 (1997) 805–811.
[35] Y.E. Joo, K.S. Seo, J. Kim, H.S. Kim, J.S. Rew, C.S. Park, S.J. Kim, J. Korean Med. Sci. 14 (1999) 417–423.
[36] E.A. Baker, F.G. Bergin, D.J. Leaper, Br. J. Surg. 87 (2000) 1215–1221.
[37] S. Yamagata, Y. Yoshii, J.G. Suh, R. Tanaka, S. Shimizu, Cancer Lett. 59 (1991) 51–55.
[38] S.L. Parsons, S.A. Watson, H.M. Collins, N.R. Griffin, P.A. Clarke, R.J. Steele, Br. J. Cancer 78 (1998) 1495–1502.
[39] I. Pucci-Minafra, S. Minafra, G. La Rocca, M. Barranca, S. Fontana, G. Alaimo, Y. Okada, Matrix Biol. 20 (2001) 419–427.
[40] E.T. Waas, R.M. Lomme, J. DeGroot, T. Wobbes, T. Hendriks, Br. J. Cancer 86 (2002) 1876–1883.
[41] S. Zucker, R.M. Lysik, M.H. Zarrabi, U. Moll, Cancer Res. 53 (1993) 140–146.
[42] A.M. Sonnante, M. Correale, M. Linsalata, A. Di Leo, V. Guerra, Scand. J. Gastroenterol. 35 (2000) 671–672.
[43] S. Zucker, R.M. Lysik, B.I. DiMassimo, H.M. Zarrabi, U.M. Moll, R. Grimson, S.P. Tickle, A.J. Docherty, Cancer 76 (1995) 700–708.
[44] S. Zucker, R.M. Lysik, M.H. Zarrabi, W. Stetler-Stevenson, L.A. Liotta, H. Birkedal-Hansen, W. Mann, M. Furie, Cancer Epidemiol. Biomark. Prev. 1 (1992) 475–479.
[45] M.N. Holten-Andersen, R.W. Stephens, H.J. Nielsen, G. Murphy, I.J. Christensen, W. Stetler-Stevenson, N. Brunner, Clin. Cancer Res. 6 (2000) 4292–4299.
[46] M.N. Holten-Andersen, G. Murphy, H.J. Nielsen, A.N. Pedersen, I.J. Christensen, G. Hoyer-Hansen, N. Brunner, R.W. Stephens, Br. J. Cancer 80 (1999) 495–503.
[47] N. Yukawa, T. Yoshikawa, M. Akaike, Y. Sugimasa, S. Takemiya, S. Yanoma, Y. Noguchi, Y. Takanashi, Br. J. Surg. 88 (2001) 1596–1601.
[48] A. Oberg, M. Hoyhtya, B. Tavelin, R. Stenling, G. Lindmark, Anticancer Res. 20 (2000) 1085–1091.
[49] P. Pellegrini, I. Contasta, A.M. Berghella, E. Gargano, C. Mammarella, D. Adorno, Cancer Immunol. Immunother. 49 (2000) 388–394.
[50] M.N. Holten-Andersen, I.J. Christensen, H.J. Nielsen, R.W. Stephens, V. Jensen, O.H. Nielsen, S. Sorensen, J. Overgaard, H. Lilja, A. Harris, G. Murphy, N. Brunner, Clin. Cancer Res. 8 (2002) 156–164.
[51] N. Okada, H. Ishida, N. Murata, D. Hashimoto, Y. Seyama, S. Kubota, Biochem. Biophys. Res. Commun. 288 (2001) 212–216.
[52] D.W. Cottam, R.C. Rees, Int. J. Oncol. 2 (1993) 861–872.
[53] S. Yamagata, Y. Ito, R. Tanaka, S. Shimizu, Biochem. Biophys. Res. Commun. 151 (1988) 158–162.
[54] V. Shah, S. Kumar, K.A. Zirvi, In Vivo 8 (1994) 321–326.
[55] J. Kishi, R. Tanaka, O. Koiwai, S. Yamagata, Y. Numata, T. Hayakawa, S. Shimizu, Cell Biol. Int. 18 (1994) 165–170.
[56] S. Hyuga, Y. Nishikawa, K. Sakata, H. Tanaka, S. Yamagata, K. Sugita, S. Saga, M. Matsuyama, S. Shimizu, Cancer Res. 54 (1994) 3611–3616.
[57] R.E. Hewitt, K.E. Brown, M. Corcoran, W.G. Stetler-Stevenson, J. Pathol. 192 (2000) 455–459.
[58] S. Mc Donnell, V. Chaudhry, J. Mansilla-Soto, Z.S. Zeng, W.P. Shu, J.G. Guillem, Clin. Exp. Metastasis 17 (1999) 341–349.
[59] D.L. Ornstein, J. MacNab, K.H. Cohn, Clin. Exp. Metastasis 17 (1999) 205–212.
[60] G. Opdenakker, P.E. Van den Steen, J. Van Damme, Trends Immunol. 22 (2001) 571–579.
[61] U. Kintscher, D. Kon, S. Wakino, S. Goetze, K. Graf, E. Fleck, W.A. Hsueh, R.E. Law, J. Cardiovasc. Pharmacol. 37 (2001) 532–539.
[62] A. Nencioni, M.R. Muller, F. Grunebach, A. Garuti, M.C. Mingari, F. Patrone, A. Ballestrero, P. Brossart, Cancer Gene Ther. 10 (2003) 209–214.
[63] M. Osman, M. Tortorella, M. Londei, S. Quaratino, Immunology 105 (2002) 73–82.
[64] P. Albertsson, M.H. Kim, L.E. Jonges, R.P. Kitson, P.J. Kuppen, B.R. Johansson, U. Nannmark, R.H. Goldfarb, In Vivo 14 (2000) 269–276.
[65] D. Leppert, E. Waubant, R. Galardy, N.W. Bunnett, S.L. Hauser, J. Immunol. 154 (1995) 4379–4389.
[66] C. Trocme, P. Gaudin, S. Berthier, C. Barro, P. Zaoui, F. Morel, J. Biol. Chem. 273 (1998) 20677–20684.
[67] J.F. Pruijt, W.E. Fibbe, L. Laterveer, R.A. Pieters, I.J. Lindley, L. Paemen, S. Masure, R. Willemze, G. Opdenakker, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 10863–10868.
[68] A. D'Haese, A. Wuyts, C. Dillen, B. Dubois, A. Billiau, H. Heremans, J. Van Damme, B. Arnold, G. Opdenakker, J. Interferon Cytokine Res. 20 (2000) 667–674.
[69] J.P. Segain, J. Harb, M. Gregoire, K. Meflah, J. Menanteau, Cancer Res. 56 (1996) 5506–5512.
[70] K. Ko, S. Yazumi, K. Yoshikawa, Y. Konda, M. Nakajima, T. Chiba, R. Takahashi, Int. J. Cancer 87 (2000) 165–171.
[71] A. Fabra, M. Nakajima, C.D. Bucana, I.J. Fidler, Differentiation 52 (1992) 101–110.
[72] C.J. Swallow, M.P. Murray, J.G. Guillem, Clin. Exp. Metastasis 14 (1996) 3–11.
[73] O.R. Mook, C. Van Overbeek, E.G. Ackema, F. Van Maldegem, W.M. Frederiks, J. Histochem. Cytochem. 51 (2003) 821–829.
[74] J. Bian, Y. Wang, M.R. Smith, H. Kim, C. Jacobs, J. Jackman, H.F. Kung, N.H. Colburn, Y. Sun, Carcinogenesis 17 (1996) 1805–1811.
[75] H. Li, F. Lindenmeyer, C. Grenet, P. Opolon, S. Menashi, C. Soria, P. Yeh, M. Perricaudet, H. Lu, Hum. Gene Ther. 12 (2001) 515–526.
[76] K. Brand, A.H. Baker, A. Perez-Canto, A. Possling, M. Sacharjat, M. Geheeb, W. Arnold, Cancer Res. 60 (2000) 5723–5730.
[77] K. Yamauchi, Y. Ogata, H. Nagase, K. Shirouzu, Surg. Today 31 (2001) 791–798.
[78] X. Wang, X. Fu, P.D. Brown, M.J. Crimmin, R.M. Hoffman, Cancer Res. 54 (1994) 4726–4728.
[79] T. Aparicio, S. Kermorgant, V. Dessirier, M.J. Lewin, T. Lehy, Carcinogenesis 20 (1999) 1445–1451.
[80] S.A. Watson, T.M. Morris, G. Robinson, M.J. Crimmin, P.D. Brown, J.D. Hardcastle, Cancer Res. 55 (1995) 3629–3633.
[81] A. Kruger, R. Soeltl, I. Sopov, C. Kopitz, M. Arlt, V. Magdolen, N. Harbeck, B. Gansbacher, M. Schmitt, Cancer Res. 61 (2001) 1272–1275.
[82] Z. An, X. Wang, N. Willmott, S.K. Chander, S. Tickle, A.J. Docherty, A. Mountain, A.T. Millican, R. Morphy, J.R. Porter, R.O. Epemolu, T. Kubota, A.R. Moossa, R.M. Hoffman, Clin. Exp. Metastasis 15 (1997) 184–195.
[83] A.H. Drummond, P. Beckett, P.D. Brown, E.A. Bone, A.H. Davidson, W.A. Galloway, A.J. Gearing, P. Huxley, D. Laber, M. McCourt, M. Whittaker, L.M. Wood, A. Wright, Ann. N.Y. Acad. Sci. 878 (1999) 228–235.
[84] T. Matsuoka, M. Yashiro, T. Sawada, T. Ishikawa, M. Ohira, K.H. Chung, Anticancer Res. 20 (2000) 4331–4338.
[85] L. Lozonschi, M. Sunamura, M. Kobari, S. Egawa, L. Ding, S. Matsuno, Cancer Res. 59 (1999) 1252–1258.

[86] K. Oba, H. Konno, T. Tanaka, M. Baba, K. Kamiya, M. Ohta, T. Kaneko, T. Shouji, A. Igarashi, S. Nakamura, Cancer Lett. 175 (2002) 45–51.
[87] R. Maekawa, H. Maki, H. Yoshida, K. Hojo, H. Tanaka, T. Wada, N. Uchida, Y. Takeda, H. Kasai, H. Okamoto, H. Tsuzuki, Y. Kambayashi, F. Watanabe, K. Kawada, K. Toda, M. Ohtani, K. Sugita, T. Yoshioka, Cancer Res. 59 (1999) 1231–1235.
[88] R. Maekawa, H. Maki, T. Wada, H. Yoshida, K. Nishida-Nishimoto, H. Okamoto, Y. Matsumoto, H. Tsuzuki, T. Yoshioka, Clin. Exp. Metastasis 18 (2000) 61–66.
[89] M. Ohta, H. Konno, T. Tanaka, M. Baba, K. Kamiya, K. Oba, T. Kaneko, T. Syouji, A. Igarashi, S. Nakamura, Jpn. J. Cancer Res. 92 (2001) 688–695.
[90] H. Maki, K. Hojo, H. Tanaka, T.Y. Sawada, R. Maekawa, T. Yoshioka, Clin. Exp. Metastasis 19 (2002) 519–526.
[91] A. Mauviel, J. Cell. Biochem. 53 (1993) 288–295.
[92] H. Qin, Y. Sun, E.N. Benveniste, J. Biol. Chem. 274 (1999) 29130–29137.
[93] J. Bian, Y. Sun, Mol. Cell Biol. 17 (1997) 6330–6338.
[94] P.R. Mertens, M.A. Alfonso-Jaume, K. Steinmann, D.H. Lovett, J. Biol. Chem. 273 (1998) 32957–32965.
[95] P.R. Mertens, K. Steinmann, M.A. Alfonso-Jaume, A. En-Nia, Y. Sun, D.H. Lovett, J. Biol. Chem. 277 (2002) 24875–24882.
[96] S. Cheng, M.A. Alfonso-Jaume, P.R. Mertens, D.H. Lovett, Biochem. J. 366 (2002) 807–816.
[97] M.R. Pan, W.C. Hung, J. Biol. Chem. 277 (2002) 32775–32780.
[98] K.R. Laderoute, J.M. Calaoagan, A.M. Knapp, H.L. Mendonca, R.S. Johnson, Biochem. Biophys. Res. Commun. 284 (2001) 1134–1139.
[99] C. Yan, H. Wang, D.D. Boyd, J. Biol. Chem. 277 (2002) 10804–10812.
[100] M.A. Davies, D. Koul, H. Dhesi, R. Berman, T.J. McDonnell, D. McConkey, W.K. Yung, P.A. Steck, Cancer Res. 59 (1999) 2551–2556.
[101] B.P. Himelstein, E.J. Lee, H. Sato, M. Seiki, R.J. Muschel, Clin. Exp. Metastasis 16 (1998) 169–177.
[102] D.L. Crowe, K.J. Tsang, B. Shemirani, Neoplasia 3 (2001) 27–32.
[103] J. Sanceau, D.D. Boyd, M. Seiki, B. Bauvois, J. Biol. Chem. 277 (2002) 35766–35775.
[104] C. Yan, H. Wang, D.D. Boyd, J. Biol. Chem. 276 (2001) 1164–1172.
[105] D.L. Crowe, T.N. Brown, Neoplasia 1 (1999) 368–372.
[106] Y. Seki, M.A. Suico, A. Uto, A. Hisatsune, T. Shuto, Y. Isohama, H. Kai, Cancer Res. 62 (2002) 6579–6586.
[107] C. Simon, H. Goepfert, D. Boyd, Cancer Res. 58 (1998) 1135–1139.
[108] C. Simon, M. Simon, G. Vucelic, M.J. Hicks, P.K. Plinkert, A. Koitschev, H.P. Zenner, Exp. Cell Res. 271 (2001) 344–355.
[109] I.K. Wang, S.Y. Lin-Shiau, J.K. Lin, Oncology 59 (2000) 245–254.
[110] S. Shimizu, Y. Nishikawa, K. Kuroda, S. Takagi, K. Kozaki, S. Hyuga, S. Saga, M. Matsuyama, Cancer Res. 56 (1996) 3366–3370.
[111] S. Hsu, F. Huang, M. Hafez, S. Winawer, E. Friedman, Cell Growth Differ. 5 (1994) 267–275.
[112] F. Huang, E. Newman, D. Theodorescu, R.S. Kerbel, E. Friedman, Cell Growth Differ. 6 (1995) 1635–1642.
[113] H. Nishibori, M. Watanabe, S. Narai, T. Kubota, C. Matsubara, T. Teramoto, M. Kitajima, Cancer Lett. 142 (1999) 83–89.
[114] I. Sehgal, T.C. Thompson, Mol. Biol. Cell 10 (1999) 407–416.
[115] R.K. Singh, M. Gutman, C.D. Bucana, R. Sanchez, N. Llansa, I.J. Fidler, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 4562–4566.
[116] S. Ozawa, H. Shinohara, H.O. Kanayama, C.J. Bruns, C.D. Bucana, L.M. Ellis, D.W. Davis, I.J. Fidler, Neoplasia 3 (2001) 154–164.
[117] S. Kermorgant, T. Aparicio, V. Dessirier, M.J. Lewin, T. Lehy, Carcinogenesis 22 (2001) 1035–1042.
[118] K. Nabeshima, T. Inoue, Y. Shimao, Y. Okada, Y. Itoh, M. Seiki, M. Koono, Cancer Res. 60 (2000) 3364–3369.
[119] A. Uchiyama, R. Essner, F. Doi, T. Nguyen, K.P. Ramming, T. Nakamura, D.L. Morton, D.S. Hoon, J. Cell. Biochem. 62 (1996) 443–453.
[120] L.J. McCawley, S. Li, E.V. Wattenberg, L.G. Hudson, J. Biol. Chem. 274 (1999) 4347–4353.
[121] G. Bellone, A. Carbone, N. Sibona, O. Bosco, D. Tibaudi, C. Smirne, T. Martone, C. Gramigni, M. Camandona, G. Emanuelli, U. Rodeck, Cancer Res. 61 (2001) 2200–2206.
[122] R. Brew, J.S. Erikson, D.C. West, A.R. Kinsella, J. Slavin, S.E. Christmas, Cytokine 12 (2000) 78–85.
[123] A. Li, M.L. Varney, R.K. Singh, Clin. Cancer Res. 7 (2001) 3298–3304.
[124] W.R. Wong, S. Kossodo, I.E. Kochevar, J. Formos. Med. Assoc. 100 (2001) 377–382.
[125] P.E. Van den Steen, B. Dubois, I. Nelissen, P.M. Rudd, R.A. Dwek, G. Opdenakker, Crit. Rev. Biochem. Mol. Biol. 37 (2002) 375–536.
[126] G.G. Vaday, H. Schor, M.A. Rahat, N. Lahat, O. Lider, J. Leukoc. Biol. 69 (2001) 613–621.
[127] L.M. Wahl, M.L. Corcoran, J. Periodontol. 64 (1993) 467–473.
[128] C.M. Klier, P.J. Nelson, Ann. N.Y. Acad. Sci. 878 (1999) 575–577.
[129] M. Locati, U. Deuschle, M.L. Massardi, F.O. Martinez, M. Sironi, S. Sozzani, T. Bartfai, A. Mantovani, J. Immunol. 168 (2002) 3557–3562.
[130] S.C. Robinson, K.A. Scott, F.R. Balkwill, Eur. J. Immunol. 32 (2002) 404–412.
[131] M.L. Corcoran, W.G. Stetler-Stevenson, P.D. Brown, L.M. Wahl, J. Biol. Chem. 267 (1992) 515–519.
[132] S. Lacraz, L.P. Nicod, R. Chicheportiche, H.G. Welgus, J.M. Dayer, J. Clin. Invest. 96 (1995) 2304–2310.
[133] A. Janowska-Wieczorek, L.A. Marquez, J.M. Nabholtz, M.L. Cabuhat, J. Montano, H. Chang, J. Rozmus, J.A. Russell, D.R. Edwards, A.R. Turner, Blood 93 (1999) 3379–3390.
[134] A. Janowska-Wieczorek, L.A. Marquez, A. Dobrowsky, M.Z. Ratajczak, M.L. Cabuhat, Exp. Hematol. 28 (2000) 1274–1285.
[135] M. Kawaguchi, R. Hosotani, S. Ohishi, N. Fujii, S.S. Tulachan, M. Koizumi, E. Toyoda, T. Masui, S. Nakajima, S. Tsuji, J. Ida, K. Fujimoto, M. Wada, R. Doi, M. Imamura, Biochem. Biophys. Res. Commun. 288 (2001) 711–717.
[136] Y. Ohnishi, H. Fujii, K. Murakami, T. Sakamoto, K. Tsukada, M. Fujimaki, M. Kojima, I. Saiki, Cancer Lett. 124 (1998) 157–163.
[137] M. Agrez, X. Gu, J. Turton, C. Meldrum, J. Niu, T. Antalis, E.W. Howard, Int. J. Cancer 81 (1999) 90–97.
[138] X. Gu, J. Niu, D.J. Dorahy, R. Scott, M.V. Agrez, Br. J. Cancer 87 (2002) 348–351.
[139] J. Niu, X. Gu, J. Turton, C. Meldrum, E.W. Howard, M. Agrez, Biochem. Biophys. Res. Commun. 249 (1998) 287–291.
[140] J.P. Annes, D.B. Rifkin, J.S. Munger, FEBS Lett. 511 (2002) 65–68.
[141] G.J. Thomas, I.R. Hart, P.M. Speight, J.F. Marshall, Br. J. Cancer 87 (2002) 859–867.
[142] N. Daemi, N. Thomasset, J.C. Lissitzky, J. Dumortier, M.F. Jacquier, C. Pourreyron, P. Rousselle, J.A. Chayvialle, L. Remy, Int. J. Cancer 85 (2000) 850–856.
[143] R. Reich, M. Blumenthal, M. Liscovitch, Clin. Exp. Metastasis 13 (1995) 134–140.
[144] R.E. Seftor, E.A. Seftor, W.G. Stetler-Stevenson, M.J. Hendrix, Cancer Res. 53 (1993) 3411–3415.
[145] A.A. Thant, A. Nawa, F. Kikkawa, Y. Ichigotani, Y. Zhang, T.T. Sein, A.R. Amin, M. Hamaguchi, Clin. Exp. Metastasis 18 (2000) 423–428.
[146] T. Sugiura, F. Berditchevski, J. Cell Biol. 146 (1999) 1375–1389.
[147] S.M. Ellerbroek, D.A. Fishman, A.S. Kearns, L.M. Bafetti, M.S. Stack, Cancer Res. 59 (1999) 1635–1641.
[148] K.M. Khan, D.J. Falcone, J. Biol. Chem. 272 (1997) 8270–8275.
[149] B. Xie, A. Laouar, E. Huberman, J. Biol. Chem. 273 (1998) 11576–11582.
[150] V.P. Yakubenko, R.R. Lobb, E.F. Plow, T.P. Ugarova, Exp. Cell Res. 260 (2000) 73–84.
[151] J. Esparza, C. Vilardell, J. Calvo, M. Juan, J. Vives, A. Urbano-Marquez, J. Yague, M.C. Cid, Blood 94 (1999) 2754–2766.

[152] K.M. Faisal Khan, G.W. Laurie, T.A. McCaffrey, D.J. Falcone, J. Biol. Chem. 277 (2002) 13778–13786.
[153] Y. Jo, J. Yeon, H.J. Kim, S.T. Lee, Biochem. J. 345 (2000) 511–519.
[154] W. Hornebeck, H. Emonard, J.C. Monboisse, G. Bellon, Semin. Cancer Biol. 12 (2002) 231–241.
[155] P.C. Brooks, S. Stromblad, L.C. Sanders, T.L. von Schalscha, R.T. Aimes, W.G. Stetler-Stevenson, J.P. Quigley, D.A. Cheresh, Cell 85 (1996) 683–693.
[156] S. Silletti, T. Kessler, J. Goldberg, D.L. Boger, D.A. Cheresh, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 119–124.
[157] H. Kolkenbrock, A. Hecker-Kia, D. Orgel, N. Ulbrich, H. Will, Biol. Chem. 378 (1997) 71–76.
[158] M. Seiki, APMIS 107 (1999) 137–143.
[159] E. Llano, A.M. Pendas, J.P. Freije, A. Nakano, V. Knauper, G. Murphy, C. Lopez-Otin, Cancer Res. 59 (1999) 2570–2576.
[160] T. Sorsa, T. Salo, E. Koivunen, J. Tyynela, Y.T. Konttinen, U. Bergmann, A. Tuuttila, E. Niemi, O. Teronen, P. Heikkila, H. Tschesche, J. Leinonen, S. Osman, U.H. Stenman, J. Biol. Chem. 272 (1997) 21067–21074.
[161] A. Lukkonen, T. Sorsa, T. Salo, T. Tervahartiala, E. Koivunen, L. Golub, S. Simon, U.H. Stenman, Int. J. Cancer 86 (2000) 577–581.
[162] A. Paju, T. Sorsa, T. Tervahartiala, E. Koivunen, C. Haglund, A. Leminen, T. Wahlstrom, T. Salo, U.H. Stenman, Br. J. Cancer 84 (2001) 1363–1371.
[163] Y. Ogata, J.J. Enghild, H. Nagase, J. Biol. Chem. 267 (1992) 3581–3584.
[164] T. Crabbe, B. Smith, J. O'Connell, A. Docherty, FEBS Lett. 345 (1994) 14–16.
[165] Q.X. Sang, H. Birkedal-Hansen, H.E. Van Wart, Biochim. Biophys. Acta 1251 (1995) 99–108.
[166] L. Devy, A. Noel, E. Baramova, K. Bajou, C. Trentesaux, J.C. Jardillier, J.M. Foidart, P. Jeannesson, Biochem. Biophys. Res. Commun. 238 (1997) 842–846.
[167] C. Festuccia, F. Guerra, S. D'Ascenzo, D. Giunciuglio, A. Albini, M. Bologna, Int. J. Cancer 75 (1998) 418–431.
[168] R. Mazzieri, L. Masiero, L. Zanetta, S. Monea, M. Onisto, S. Garbisa, P. Mignatti, EMBO J. 16 (1997) 2319–2332.
[169] R. Fridman, M. Toth, D. Pena, S. Mobashery, Cancer Res. 55 (1995) 2548–2555.
[170] H. Nakamura, Y. Fujii, E. Ohuchi, E. Yamamoto, Y. Okada, Eur. J. Biochem. 253 (1998) 67–75.
[171] V. Knauper, B. Smith, C. Lopez-Otin, G. Murphy, Eur. J. Biochem. 248 (1997) 369–373.
[172] J.A. Uria, C. Lopez-Otin, Cancer Res. 60 (2000) 4745–4751.
[173] T. Crabbe, C. Ioannou, A.J. Docherty, Eur. J. Biochem. 218 (1993) 431–438.
[174] H. Emonard, W. Hornebeck, Biol. Chem. 378 (1997) 265–271.
[175] G.A. Bannikov, T.V. Karelina, I.E. Collier, B.L. Marmer, G.I. Goldberg, J. Biol. Chem. 277 (2002) 16022–16027.
[176] G. Bergers, L.M. Coussens, Curr. Opin. Genet. Dev. 10 (2000) 120–127.
[177] Q. Yu, I. Stamenkovic, Genes Dev. 14 (2000) 163–176.
[178] C. Fernandez-Patron, C. Zouki, R. Whittal, J.S. Chan, S.T. Davidge, J.G. Filep, FASEB J. 15 (2001) 2230–2240.
[179] M.A. Lombard, T.L. Wallace, M.F. Kubicek, G.L. Petzold, M.A. Mitchell, S.K. Hendges, J.W. Wilks, Cancer Res. 58 (1998) 4001–4007.
[180] E. Levi, R. Fridman, H.Q. Miao, Y.S. Ma, A. Yayon, I. Vlodavsky, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7069–7074.
[181] G.A. McQuibban, J.H. Gong, E.M. Tam, C.A. McCulloch, I. Clark-Lewis, C.M. Overall, Science 289 (2000) 1202–1206.
[182] B.C. Sheu, S.M. Hsu, H.N. Ho, H.C. Lien, S.C. Huang, R.H. Lin, Cancer Res. 61 (2001) 237–242.
[183] L. Gorelik, R.A. Flavell, Nat. Med. 7 (2001) 1118–1122.
[184] S.J. Kridel, E. Chen, L.P. Kotra, E.W. Howard, S. Mobashery, J.W. Smith, J. Biol. Chem. 276 (2001) 20572–20578.
[185] M.W. Olson, M. Toth, D.C. Gervasi, Y. Sado, Y. Ninomiya, R. Fridman, J. Biol. Chem. 273 (1998) 10672–10681.
[186] E. Fiore, C. Fusco, P. Romero, I. Stamenkovic, Oncogene 21 (2002) 5213–5223.
[187] U. Schonbeck, F. Mach, P. Libby, J. Immunol. 161 (1998) 3340–3346.
[188] T. Itoh, M. Tanioka, H. Yoshida, T. Yoshioka, H. Nishimoto, S. Itohara, Cancer Res. 58 (1998) 1048–1051.
[189] G. Bergers, R. Brekken, G. McMahon, T.H. Vu, T. Itoh, K. Tamaki, K. Tanzawa, P. Thorpe, S. Itohara, Z. Werb, D. Hanahan, Nat. Cell Biol. 2 (2000) 737–744.
[190] H.J. Mauceri, S. Seetharam, M.A. Beckett, J.Y. Lee, V.K. Gupta, S. Gately, M.S. Stack, C.K. Brown, K. Swedberg, D.W. Kufe, R.R. Weichselbaum, Int. J. Cancer 97 (2002) 410–415.
[191] A. Pozzi, W.F. LeVine, H.A. Gardner, Oncogene 21 (2002) 272–281.
[192] M. Ferreras, U. Felbor, T. Lenhard, B.R. Olsen, J. Delaisse, FEBS Lett. 486 (2000) 247–251.
[193] Y.M. Kim, J.W. Jang, O.H. Lee, J. Yeon, E.Y. Choi, K.W. Kim, S.T. Lee, Y.G. Kwon, Cancer Res. 60 (2000) 5410–5413.
[194] P.C. Brooks, S. Silletti, T.L. von Schalscha, M. Friedlander, D.A. Cheresh, Cell 92 (1998) 391–400.
[195] S. Zucker, J. Cao, W.T. Chen, Oncogene 19 (2000) 6642–6650.
[196] S.J. Kridel, E. Chen, L.P. Kotra, E.W. Howard, S. Mobashery, J.W. Smith, J. Biol. Chem. 276 (2001) 20572–20578.
[197] E.I. Chen, S.J. Kridel, E.W. Howard, W. Li, A. Godzik, J.W. Smith, J. Biol. Chem. 277 (2002) 4485–4491.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 48, Issue of November 28, pp. 47654–47659, 2003
Printed in U.S.A.

Stanniocalcin 1 Is an Autocrine Modulator of Endothelial Angiogenic Responses to Hepatocyte Growth Factor*

Received for publication, February 6, 2003, and in revised form, July 24, 2003
Published, JBC Papers in Press, September 17, 2003, DOI 10.1074/jbc.M301353200

Constance Zlot‡, Gladys Ingle‡, Joanne Hongo§, Suya Yang¶, Zhong Sheng¶, Ralph Schwall¶, Nicholas Paoni‡, Fay Wang‡, Franklin V. Peale, Jr.‖, and Mary E. Gerritsen‡**

From the Departments of ‡Cardiovascular Research, §Antibody Technology, ¶Molecular Oncology, and ‖Pathology, Genentech Inc., South San Francisco, California 94080

Stanniocalcin 1 (STC1) is a secreted glycoprotein originally described as a hormone involved in calcium and phosphate homeostasis in bony fishes. We recently identified the mammalian homolog of this molecule to be highly up-regulated in an *in vitro* model of angiogenesis, as well as focally and intensely expressed at sites of pathological angiogenesis (*e.g.* tumor vasculature). In the present study, we report that STC1 is a selective modulator of hepatocyte growth factor (HGF)-induced endothelial migration and morphogenesis, but not proliferation. STC1 did not inhibit proliferative or migratory responses to vascular endothelial growth factor or basic fibroblast growth factor. The mechanism of STC1 inhibitory effects on HGF-induced endothelial migration seem to occur secondary to receptor activation because STC1 did not inhibit HGF-induced c-met receptor phosphorylation, but did block HGF-induced focal adhesion kinase activation. In the mouse femoral artery ligation model of angiogenesis, STC1 expression closely paralleled that of the endothelial marker CD31, and the peak level of STC1 expression occurred after an increase in HGF expression. We propose that STC1 may play a selective modulatory role in angiogenesis, possibly serving as a "stop signal" or stabilizing factor contributing to the maturation of newly formed blood vessels. HGF is a mesenchyme-derived pleiotropic factor with mitogenic, motogenic, and morphogenic activities on a number of different cell types. HGF effects are mediated through a specific tyrosine kinase, *c-met*, and aberrant HGF and *c-met* expression are frequently observed in a variety of tumors. Recent studies have shown HGF to be a potent growth factor implicated in wound healing, tissue regeneration, and angiogenesis.

We recently reported that HGF,[1] and more potently, HGF in combination with VEGF, synergistically induced vascular morphogenesis *in vitro* and angiogenesis *in vivo* (1). In a related study, we analyzed the gene expression profile of endothelial cells undergoing HGF- and VEGF-stimulated morphogenesis using Affymetrix oligonucleotide arrays. We identified the homodimeric secreted glycoprotein, stanniocalcin-1 (STC1), as one of the most highly up-regulated genes in this *in vitro* model (2). We also observed intense expression of STC1 in the vasculature of colon carcinomas, providing further evidence that this novel glycoprotein might play an important role in one or more of the processes associated with angiogenesis (2).

The objective of the present study was to determine what role STC1 played in HGF-induced responses of vascular endothelial cells.

MATERIALS AND METHODS

Reagents—Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA) and maintained in endothelial growth medium (Clonetics) supplemented with fetal bovine serum to a final concentration of 10%. Other reagents included type 1 rat tail collagen (Upstate Biotechnology, Lake Placid, NY), recombinant bFGF (Collaborative Biomedical Products, Bedford, MA), and recombinant VEGF and HGF (Genentech, South San Francisco, CA). All other cell culture reagents were obtained from Invitrogen.

Isolation of STC1 and Construction of Expression Vectors—cDNA clones were isolated from a human endothelial cDNA library and sequenced in their entirety. Fc fusion proteins (immunoadhesins) were prepared by fusion of the entire open reading frame of STC-1 in frame with the Fc region of human IgG1 using the baculovirus vector pHIF, a derivative of pVL1393 purchased from PharMingen. Fusion proteins were transiently expressed in Sf9 insect cells and purified over a protein A column. STC1 was also expressed as a C-terminal His tag fusion in *Escherichia coli* and the denatured protein was used for immunization. The identities of the purified proteins were verified by N-terminal sequence analysis.

Preparation of Monoclonal Antibodies to STC1—Ten *Balb/c* mice (Charles River Laboratories, Wilmington, DE) were hyperimmunized with recombinant polyhistidine-tagged (HIS8) human STC1 expressed in *E. coli* (Genentech) in Ribi adjuvant (Ribi Immunochem Research, Inc., Hamilton, MO). B-cells from five mice demonstrating high anti-STC1 antibody titers were fused with mouse myeloma cells (X63.Ag8.653; American Type Culture Collection, Rockville, MD) using a modified protocol analogous to one described previously (3, 4). After 10–14 days, the supernatants were harvested and screened for antibody production by direct enzyme-linked immunosorbent assay (ELISA). Five positive clones, showing the highest immunobinding after the second round of subcloning by limiting dilution, were injected into Pristane primed mice (5) for *in vivo* production of the monoclonal antibody. The ascites fluids were pooled and purified by protein A affinity chromatography (fast protein liquid chromatography, Pharmacia, Uppsala, Sweden) as described previously (3). The purified antibody preparations were sterile filtered (0.2-μm pore size; Nalgene, Rochester, NY) and stored at 4 °C in phosphate-buffered saline. For ELISA for STC1, high binding, flat-bottom polypropylene 96-well plates (NUNC, Naperville, IL) were coated overnight at 4 °C with 100 μl monoclonal STC1 antibody 2734 (250 ng/ml). The plates were washed (phosphate-buffered saline containing 0.05% Tween), blocked (phosphate-buffered saline containing 0. 5% BSA), and washed again before adding 100 μl of supernatant or STC1 onto duplicate wells. After subsequent washing steps, a second biotinylated STC1 monoclonal antibody (2733; 250 ng/ml) was added to the wells. After a 2-h incubation and a wash step, a 1:10,000 dilution of streptavidin-horseradish peroxidase (Amersham Biosciences) was added to the plates. Tetramethyl benzidine (Kirke-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** Present address and to whom correspondence should be addressed: 541 Parrott Dr., San Mateo, CA 94402. Tel.: 650-348-6492; E-mail: meg570@comcast.net.

[1] The abbreviations used are: HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; STC1, stanniocalcin-1; HUVEC, human umbilical vein endothelial cells; ELISA, enzyme-linked immunosorbent assay; bFGF, basic fibroblast growth factor; rSTC1, Ig fusion protein.

gaard & Perry, Gaithersburg, MD) and 1 M phosphoric acid were added, and the absorbance at 450 nm was determined (Spectra Max 250, Molecular Devices, Sunnyvale, CA). The minimum level of STC1 that could be reliably detected by the ELISA was 20 pg/ml. For RNA isolation and quantitative reverse transcriptase-PCR (ABI PRISM TaqMan), Tri-reagent-LS (Molecular Research Center, Cincinnati, OH) was added to the cells, and total RNA was extracted according to manufacturer's protocols. Gene-specific oligonucleotide primer pairs and a specific probe (labeled with a fluorescent dye at the 5′ end and a quencher fluorescent dye at the 3′ end) were designed using Oligo version 4.0 software (National Biosciences, Plymouth, MN) and levels of STC1 mRNA were determined by real-time quantitative PCR (ABI PRISM TaqMan), as described previously (6).

Culture of Cells—HUVECs were routinely grown on gelatin-coated (1 μg/ml) plates in endothelial growth media. Drugs and growth factors were added to the media and pre-warmed to 37 °C before addition to the HUVECs. Collagen gels containing HUVECs were prepared as described previously (1). The gels were overlaid with 1× basal media (Medium 199 supplemented with 1% fetal bovine serum, 1% ITS (insulin, selenium and transferrin, Invitrogen), and 2 mM L-glutamine), 100 units/ml penicillin, and 100 units/ml streptomycin containing 200 ng/ml HGF and 200 ng/ml VEGF to elicit tube formation, as described previously (1). For "film" experiments, endothelial cells were seeded onto the surface of a collagen gel and incubated in the identical media as that described for the gel experiments. To evaluate endothelial morphogenesis on Matrigel™, cells were incubated in 1× basal media in the presence of various factors, as described. Network formation was quantitated at 8 h by photographing three random fields of each well and then determining the total network area/field using OpenLab software (Improvision).

Cell Migration Assay—HTS multi-well insert 24-well plates (BD Biosciences) were coated with cell attachment factor (BD Biosciences) on the bottom layer and type 1 collagen on the membrane surface. 25,000 cells were seeded into each chamber and incubated for 18 h at 37 °C in 5% CO_2. The collagen and unmigrated cells were scraped off the membrane surface with a plastic pasteur pipette, and then all media were aspirated. Absolute methanol was added to the membranes, and the membranes were fixed at room temperature for 30 min. The methanol was aspirated off, and a 10 μM solution of YO-PRO-1 (Molecular Probes, Eugene, OR) was added. Cells were counted under fluorescein isothiocyanate optics using OpenLab version 2.5 (Improvision).

Proliferation Assay—5,000 cells were seeded onto gelatin-coated 96-well plates and incubated overnight with endothelial growth medium. The cells were then starved for 3 days with M199 containing 1% fetal bovine serum, 2 mmol/liter L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. 20 ng/ml of various growth factors were added to the starvation media, and the cells were incubated for 4 days. Alamar blue solution (BIOSOURCE International, Camarillo, CA) was added to the wells in an amount equal to 10% of the culture volume and incubated for 4–6 h at 37 °C in 5% CO_2. The plates were read on a Spectra Max Gemini (Molecular Devices), with the OD excitation at 535 nm and emission at 590 nm.

Femoral Ligation Surgery—Femoral artery ligation was performed under isoflurane (Aerrane, Fort Dodge, CO) inhalation anesthesia on 8- to 10-week-old male C57/Bl6J mice (Charles River Laboratories). Briefly, the femoral artery was isolated at the level of the inguinal ligament and ligated with 7–0 silk suture (Ethicon, Somerville, NJ). Animals were allowed to recover on a warm water heating pad until ambulatory. Total RNA was isolated from the gastrocnemius muscle of both the ligated and sham animals. Six animals were used for the control (sham) and six animals for the ligated group for each time point.

Effects of STC1 on HGF-induced c-met and FAK Phosphorylation—Confluent HUVEC were incubated overnight in basal medium. Cells were pretreated with native or boiled STC1 (5 μg/ml) for 30 min. HGF was then added, and cells were incubated for a 15-min incubation at 37 °C. After the addition of lysate buffer (phosphate-buffered saline supplemented with 1% Triton, protease inhibitor mix (Sigma) and phosphatase inhibitor mix (Sigma)), lysates were immunoprecipitated with an antibody to c-met (C-28, Santa Cruz Biotechnology) and then immunoblotted for phosphotyrosine (4G10, Upstate Biotech). In other experiments, lysates were prepared after pretreatment of cells with STC1, followed by HGF, VEGF, or bFGF, and lysates were prepared as above. Samples were immunoprecipitated with an antibody to FAK (C-20, Santa Cruz Biotechnology) and then immunoblotted for phosphotyrosine as above. For the data analysis, numerical data are expressed as the mean ± S.E., and the n for each experiment is provided in the figure legends or text. To determine statistical significance, data were first evaluated by ANOVA, followed by a Student's t test for non-paired values. In experiments where multiple comparisons were made against controls, Bonferroni's modified t test was used. $p < 0.05$ was accepted as significant.

TABLE I

Effects of various agonists on the release of STC-1 by HUVEC

HUVEC monolayers were incubated with the indicated agonists, and aliquots were removed at 24, 48, and 72 hr and analyzed for STC-1 by ELISA. Data shown are the mean values (n = 4).

Stimuli	STC1		
	24 hr	48 hr	72 hr
		ng/ml	
Media	<0.02	<0.02	<0.02
Calcium ionophore (10 mg/ml)	<0.02	<0.02	ND[a]
Bradykinin (1 μM)	<0.02	<0.02	ND
Histamine (1 μM)	<0.02	<0.02	ND
VEGF (400 ng/ml)	<0.02	<0.02	<0.02
TGF β (50 ng/ml)	<0.02	<0.02	<0.02
IFNγ (25 ng/ml)	<0.02	<0.02	<0.02
bFGF (400 ng/ml)	<0.02	<0.02	.092
HGF (400 ng/ml)	<0.02	<0.02	.112
TNFα (10 ng/ml)	<0.02	<0.02	.06
IL-6 (25 ng/ml)	<0.02	.023	.114
IL-1 (10 ng/ml)	<0.02	.027	.027
IL-4 (25 ng/ml)	<0.02	.025	.122
PMA (100 ng/ml)	.023	.057	.183

[a] ND, not determined.

RESULTS

Regulation of STC1 Production in Endothelial Cells in Monolayer Culture—To evaluate the effects of various cytokines and growth factors on the release of STC1 from HUVEC, we developed an ELISA-based assay (see "Materials and Methods" for details) which was capable of measuring STC1 levels as low as 20 pg/ml. There was no detectable STC1 release from unstimulated HUVEC (not shown). To survey for the possible effects of various cytokines and growth factors on STC1 release, confluent endothelial cells cultured in 96-well tissue culture plates were incubated with these factors for 24, 48, and 72 h, and STC1 levels were determined in the cell supernatants. The majority of factors evaluated had either no effect (*e.g.* VEGF, TGF, bradykinin, histamine, and TNF) or very modest effects (IL-1) on STC1 release at these time points. The concentrations shown in Table I are the highest concentration tested (for each drug we tested, the indicated dose and at least two lower doses (*e.g.* 1:10 and 1:100) of that shown). Of the growth factors examined, only bFGF and HGF stimulated significant STC1 release. The cytokines IL-6 and IL-4 also stimulated STC1 release.

Levels of STC1 mRNA and Protein Secretion Are Much Greater in Three-dimensional Cultures Compared with Two-dimensional Cultures—Equal numbers (1.5×10^7) of HUVEC were plated either onto type I collagen gels (film experiments) or suspended in type I collagen gels (gel experiments) and incubated with HGF and VEGF (200 ng/ml) for various times as indicated in Fig. 1. It should be noted that the combination of HGF and VEGF was required in three-dimensional gels for survival; as described previously, neither growth factor alone was capable of supporting survival and tubulogenesis in three-dimensional collagen gels (1). The mRNA levels (Fig. 1*A*) for STC1 rose dramatically in the gel *versus* film environment, such that at 24 h, the mRNA levels for STC1 were 10- to 20-fold higher in the three-dimensional cultures. Levels of STC1 protein (Fig. 1*B*) in the supernatants were 2- to 6-fold higher in the three-dimensional cultures. Because the cells are embedded in three-dimensional collagen gels, any protein in the supernatants must be "released" from the gel to be detected, and this may account for the discrepancy in relative ratios of mRNA *versus* protein in the two environments.

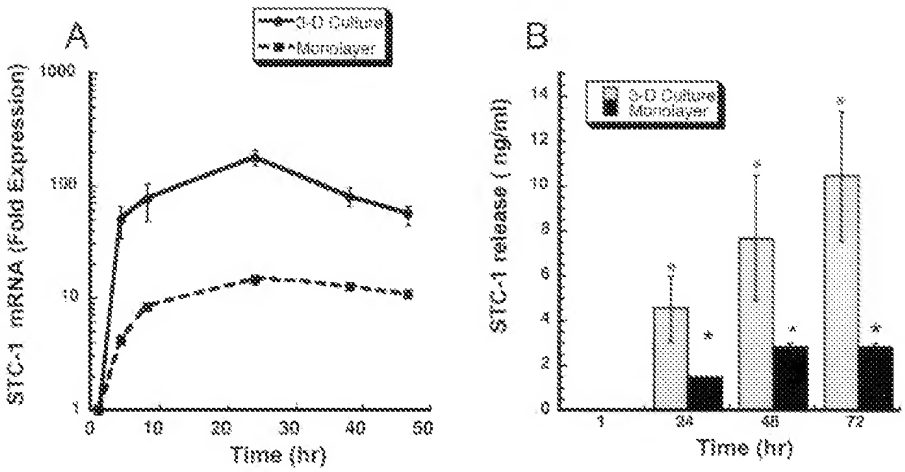


FIG. 1. **STC1 mRNA and protein are selectively up-regulated in three-dimensional gel environments.** *A*, STC1 mRNA from HUVECs cultured in three-dimensional or on the surface of monolayer collagen gels in BM supplemented with HGF (200 ng/ml) and VEGF (200 ng/ml). Duplicate samples were analyzed by quantitative RT-PCR (TaqMan), as described under "Materials and Methods." Results are expressed as the ratio of STC1 mRNA level to the level of cyclophilin, a housekeeping gene, in the same sample. *B*, STC1 protein from HUVECs cultured in three-dimensional or on the surface of monolayer collagen gels in BM supplemented with HGF (200 ng/ml) and VEGF (200 ng/ml). STC1 protein was determined by ELISA, as described under "Materials and Methods." Values shown are the mean ± S.E.; $n = 8$. *, significantly different from value at $t = 0$; $p < 0.05$.

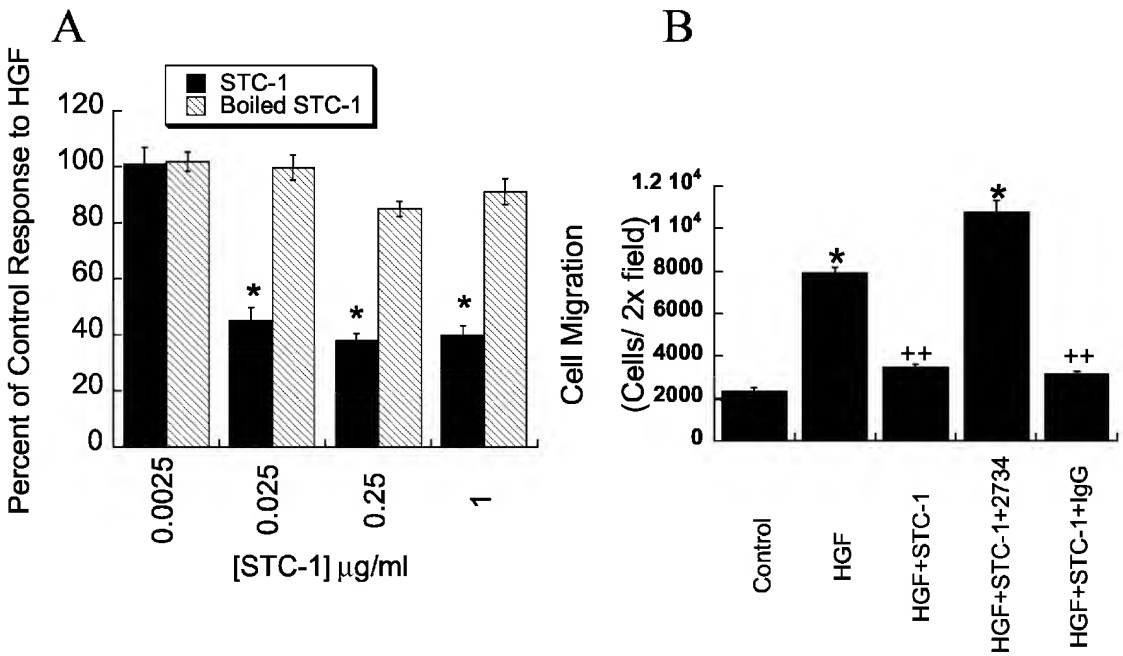


FIG. 2. **STC1 inhibits endothelial cell migration.** *A*, the migratory response of HUVEC to 20 ng/ml of HGF in the presence of native (*solid bars*) or boiled rSTC1 (*hatched bars*) at the indicated concentrations was determined. Data are expressed as the percent of the HGF control cell migration index and are the mean ± S.E., $n = 4$. Data shown are representative of three independent experiments. *, significantly different from HGF alone; $p < 0.05$. *B*, the inhibitory effects of STC1 on HGF-induced (20 ng/ml) migration are blocked by the monoclonal antibody 2734 (25 μg/ml) but not by an isotype-matched nonimmune IgG. Data are expressed as cell migration index (number of cells/2 × field) and are the mean ± S.E., $n = 4$. Data shown are representative of three independent experiments. *, significantly different from control; ++, significantly different from HGF alone; $p < 0.05$.

Effects of STC1 on Endothelial Proliferation—STC1 (0.001–5 μg/ml) had no effect on bFGF-stimulated (10 ng/ml), VEGF-stimulated (10 ng/ml), or HGF-stimulated (10 ng/ml) endothelial proliferation (data not shown). Additionally, STC1 did not stimulate endothelial proliferation when tested in the absence of growth factors (not shown).

Effects of STC1 on HGF-induced Endothelial Migration—HGF is a known potent stimulus for endothelial migration. Therefore, we determined the role of STC1 in HGF-induced endothelial cell migration. As shown in Fig. 2*A*, when rSTC1 (Ig fusion protein) was added to the Boyden chambers, it markedly inhibited the migratory response of the endothelial cells to HGF. Denaturation of the recombinant protein by boiling completely eliminated this inhibitory activity. Additionally, the inhibitory effects of STC1 were not observed at lower (2.5 ng/ml) concentrations of the protein. The inhibitory effects of STC1 on HGF cell migration were also blocked by the inclusion of 25 μg/ml of the anti-STC1 monoclonal, 2734 (Fig. 2*B*), in contrast to the lack of effect of an isotype-matched non-immune IgG. To further evaluate the selective effects of STC1 on endothelial cell migration, we evaluated the effects of rSTC1 on bFGF-induced (10 ng/ml) and VEGF-induced (10 ng/ml) endothelial cell migration. These doses of bFGF and VEGF elicited a similar magnitude of cell migration as 20 ng/ml HGF, yet none of the STC1 reagents tested (native STC1, boiled STC1, STC1 monoclonal antibody 2734 (25 μg/ml)) had a significant

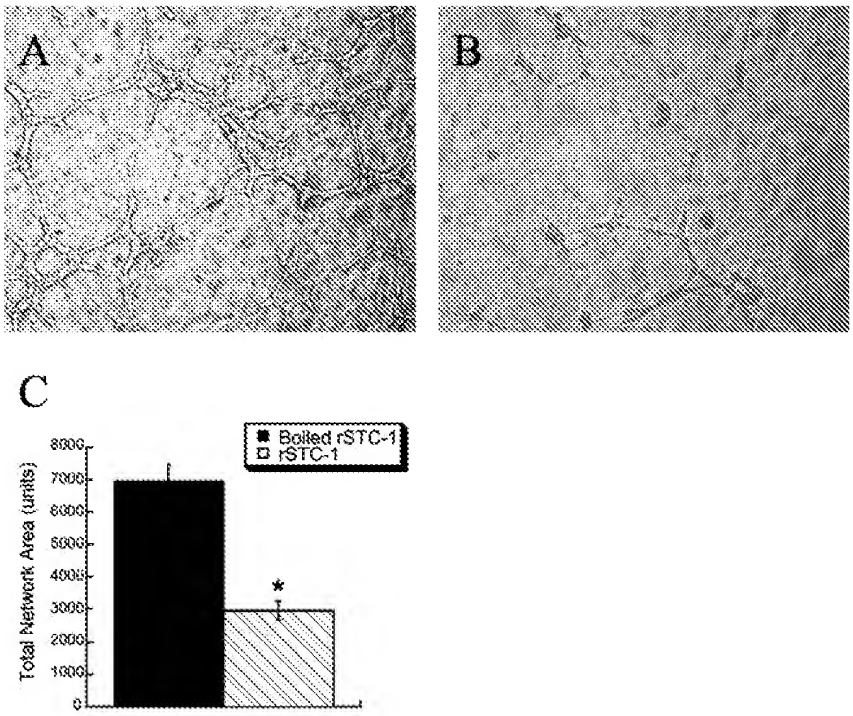


FIG. 3. **STC1 reduces endothelial cord formation on Matrigel™ induced by 20 ng/ml HGF.** *A*, HGF + boiled STC1 (250 ng/ml) *B*, HGF + native STC1 (250 ng/ml) *C*, quantitation of cord formation in the presence of boiled STC1 (*solid bars*) or native STC1 (*hatched bars*). Data shown are the mean network area/well of three independent experiments. *, significantly different from boiled control.

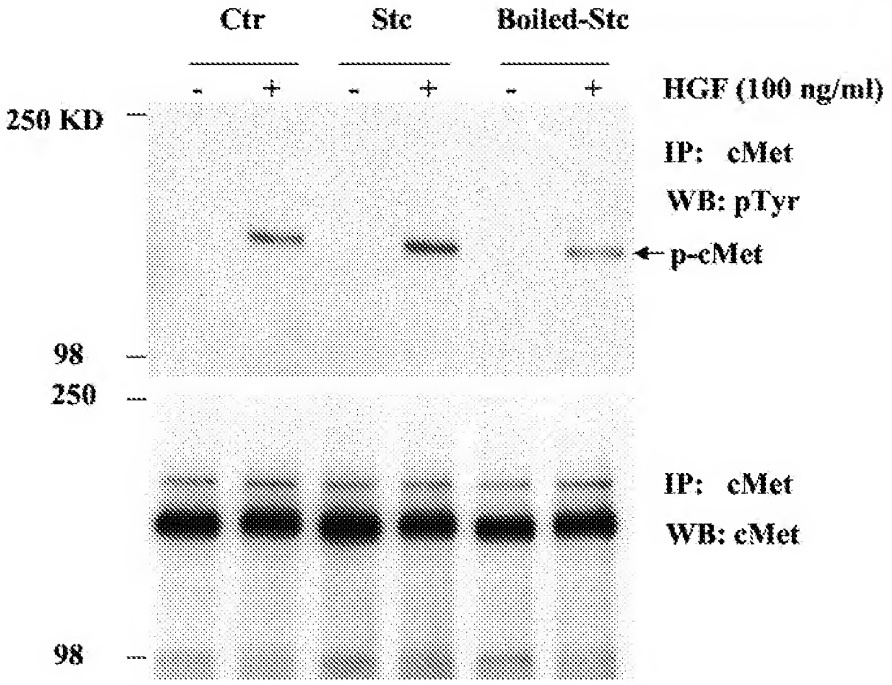


FIG. 4. **STC1 does not inhibit HGF-induced *c-met* phosphorylation.** HUVEC were pretreated for 30 min with 5 μg/ml STC1 or 5 μg/ml boiled STC1 and then challenged with HGF (100 ng/ml) for 15 min. Lysates were subjected to immunoprecipitation for *c-met*, separated by gel electrophoresis, and transferred to nitrocellulose. The resulting blots were immunoblotted with antibodies to phosphotyrosine (*pTyr*) (*top*) or *cMet* (*bottom*).

effect on the migratory response to bFGF or VEGF (data not shown).

Effects of STC1 on HGF-induced Endothelial Morphogenesis—To evaluate the possible effects of STC1 on endothelial morphogenesis, we tested the effects of native rSTC1 and boiled STC1 on HGF-induced endothelial branching network formation on growth factor-depleted Matrigel™. In this model, incubation of endothelial cells without a growth factor such as HGF results in little or no cord formation (not shown). Addition of HGF (20 ng/ml) results in an elaborate network of endothelial branching structures (Fig. 3*A*). Addition of rSTC1 (1 μg/ml) markedly reduced the overall area of endothelial networks (Fig. 3*B*), and the structures that did form were discontinuous and poorly formed. Heat denaturation abrogated the effects of rSTC1 (Fig. 3*A*).

Effects of STC1 on HGF-induced c-met and FAK Phosphorylation—HGF induced the phosphorylation of c-met (Fig. 4). Pretreatment or cotreatment of HUVEC with 5 μg/ml STC1 did not reduce the phosphorylation response of *c-met* to added HGF (Fig. 4), suggesting that STC1 did not inhibit HGF binding to its receptor. To determine possible effects downstream of c-met phosphorylation, we examined the effects of STC1 HGF-induced FAK activation. STC1 abrogated HGF-induced phosphorylation of FAK (Fig. 5). Preliminary experiments indicated that maximal FAK phosphorylation after HGF treatment occurred at 60 min. Pretreatment of the endothelial cells with 5 μg/ml STC1 completely blocked HGF-induced FAK phosphorylation. Although difficult to detect, the modest FAK phosphorylation induced by bFGF or VEGF (Fig. 5) was not inhibited by pretreatment with STC1.

Expression of STC1 in the Hind-limb Ischemia Model of Angiogenesis—We also evaluated the expression of STC1 in the mouse femoral ligation assay. In this model, the femoral artery is ligated, dramatically reducing blood flow to the lower limb including the gastrocnemius muscle (7). This hypoxic insult results in the rapid development of new vessels in the hypoxic muscle as a component of the spontaneous recovery of perfusion, which is also associated with proximal arteriogenesis (8, 9). Because the newly developing vessels are not readily visualized in this assay, we also measured the mRNA levels of the endothelial marker, CD31, as an index of new vessel formation. As shown in Fig. 6, *A* and *B*, both CD31 mRNA and STC1 mRNA expression peaked at day 3 and then returned to lower levels at later time points. We also measured the mRNA levels of HGF. Interestingly, the expression of HGF also increased markedly in this *in vivo* model, peaking at day 3 (Fig. 6*B*)

DISCUSSION

Stanniocalcin 1, a secreted protein, was first identified in fish, where it has been shown to regulate calcium and phosphate homeostasis (10, 11). A unique feature of STC1 is its lack of homology to any other proteins, except for stanniocalcin 2, to which it is 34% identical at the amino acid level. The human homolog of STC1 is 73% homologous to the salmon protein. However, in contrast to the fish, where STC1 is exclusively localized to the organ of Stannius, the mammalian homolog exhibits a much broader expression profile. The function of the mammalian STC1 is poorly understood, although roles in calcium and phosphate homeostasis (12–14) and ovarian function (15) have been suggested.

We first identified STC1 as one of the genes that demonstrated marked up-regulation in endothelial cells undergoing tubulogenesis (6), suggesting a possible role in angiogenesis. *In situ* studies demonstrated that the expression of STC1 was highly focal; high levels of expression were observed in small to large vessels at the periphery of lung and colon carcinomas and inflamed appendix (2, 6). This hypothesis gained further support upon examination of the phenotype of STC1 transgenic mice (using a muscle-specific promoter). The STC1 mice were smaller than their wild-type littermates, yet baseline organ vascularity as well as induction of increased vascular density after femoral ligation were enhanced (14). In the present study, we report that STC1 is an autocrine modulator of HGF-induced endothelial migration and morphogenesis (cord formation) on Matrigel™. These effects were selective to HGF because the responses of endothelial cells to either VEGF or bFGF were not modulated in these *in vitro* assays. Of the growth factors and cytokines examined, HGF was the most potent inducer of STC1 secretion. Finally, in an *in vivo* model of physiological angiogenesis, the mouse femoral ligation model, the expression profile of STC1 mRNA was similar to that of the endothelial marker, CD31, and moreover, the peak expression of STC1 mRNA was preceded by peak expression of HGF.

The mechanism of the selective inhibition of HGF action on endothelial cells seems to be downstream of HGF binding to and activation of its receptor, c-met. Focal adhesion kinase, a 125-kDa cytoplasmic tyrosine kinase that is localized in focal adhesions, has been shown to play an important role in integrin-mediated cellular functions, including cell spreading and migration (16–19). Recent studies have also shown that FAK activation is also linked to HGF-induced cell motility (20). FAK interacts with several intracellular signaling molecules including Src family kinases (20, 21), phosphatidylinositol 3-kinase (22), the adapter protein Grb2 (23), and the docking protein p130Cas (24), thus linking activation of this kinase to other signaling cascades. STC1 reduced HGF-induced FAK phosphorylation, consistent with hypothesis that STC1 interferes with one or more downstream signaling pathways activated by the c-met receptor. Moreover, the effects of STC1 seem to be selective for HGF because bFGF- and VEGF-induced FAK phosphorylation were not inhibited. Our data are consistent with a modulatory role of STC1 in angiogenesis, possibly serving as a stop signal or stabilization factor contributing to the maturation of newly formed blood vessels. HGF is a potent although not endothelial-selective endothelial mitogen, morphogen, and motogen (25, 26), and the expression of both HGF and its receptor c-met are known to be up-regulated in both physiological (27, 28) and pathological angiogenesis (29, 30).

STC1 receptors, at least in the liver and kidney, are present both on the plasma membrane and in the mitochondria (31). Furthermore, despite being a secreted protein, STC1 is sequestered in the mitochondria (31) and has been proposed to play a

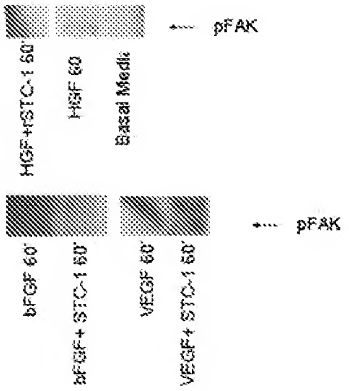


FIG. 5. **STC1 inhibits FAK phosphorylation induced by HGF.** HUVECs were pretreated for 30 min with 5 μg/ml STC1 and then challenged with HGF (10 ng/ml), VEGF (10 ng/ml), or bFGF (10 ng/ml) for 60 min. Lysates were subjected to immunoprecipitation for FAK, separated by gel electrophoresis, and transferred to nitrocellulose. The resulting blots were immunoblotted with antibodies to phosphotyrosine.

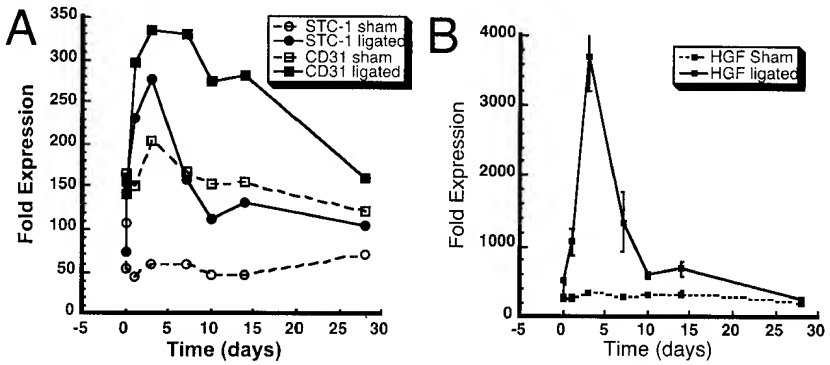


FIG. 6. **STC1 is up-regulated in the hind limb ischemia model of angiogenesis.** *A*, STC1 and CD31 mRNA in gastrocnemius muscles removed from mice after sham surgery or femoral ligation. *B*, HGF mRNA in gastrocnemius muscles removed from mice after sham surgery or femoral ligation. Values are expressed as the mean ± S.E.; $n = 6$ mice/time point and treatment. *, significantly different from sham at the same time point; $p < 0.05$.

role in the regulation of cellular metabolism (14, 31). Thus the elevated expression of STC1 during angiogenesis may play an additional role in the metabolic requirements of endothelial cells and other cells involved in the formation of new blood vessels.

Acknowledgments—We thank Hope Steinmetz and John Hoeffel for the preparation and measurement of mRNA in the gastrocnemius muscle from the mouse femoral ligation assays, Austin Gurney, Jessica Foster, Richard Vandlen, and Christopher Grimaldi for their help in preparation of recombinant STC1 1, Phil Haas, Mark Nagel, and Dan Eaton for their help in purification of the recombinant protein, and Angela Spaulding for purification of the STC1 monoclonal antibodies. We also thank Shouchun Liu for helpful comments.

REFERENCES

1. Xin, X., Yang, S., Ingle, G., Zlot, C., Rangell, L., Kowalski, J., Schwall, R., Ferrara, N., and Gerritsen, M. E. (2001) *Am. J. Pathol.* **158,** 1111–1120
2. Gerritsen, M. E., Peale, F. V., Jr., and Wu, T. (2002) *Exp. Nephrol.* **10,** 114–119
3. Hongo, J., Mora-Worms, M., Lucas, C., and Fendly, B. (1995) *Hybridoma* **14,** 253–259
4. Kohler, G., and Milstein, C. (1975) *Nature* **256,** 495–497
5. Freund, Y., and Blair, P. (1982) *J. Immunol.* **129,** 2826–2830
6. Kahn, J., Mehraban, F., Ingle, G., Xin, X., Bryant, J., Vehar, G., Schoenfeld, J., Grimaldi, C., Peale, F., Drakharapu, A., Lewin, D., and Gerritsen, M. (2000) *Am. J. Pathol.* **156,** 1887–1900
7. Couffinhal, T., Silver, M., Zheng, L. P., Kearney, M., Witzenbichler, B., and Isner, J. M. (1998) *Am. J. Pathol.* **152,** 1667–1679
8. Ito, W. D., Arras, M., Scholz, D., Winkler, B., Htun, P., and Schaper, W. (1997) *Am. J. Physiol.* **273,** 3 Pt 2, H1255–H1265
9. Scholz, D., Ito, W., Fleming, I., Deindl, E., Sauer, A., Wiesnet, M., Busse, R., Schaper, J., and Schaper, W. (2000) *Virchows Arch.* **436,** 257–270
10. Wagner, G., Hampong, M., Park, C., and Copp, D. (1986) *Gen. Comp. Endocrinol.* **63,** 481–491
11. Wagner, G. F., Dimattia, G. E., Davie, J. R., Copp, D. H., and Friesen, H. G. (1992) *Mol. Cell. Endocrinol.* **90,** 7–15
12. Wagner, G. F., Vozzolo, B. L., Jaworski, E., Haddad, M., Kline, R. L., Olsen, H. S., Rosen, C. A., Davidson, M. B., and Renfro, J. L. (1997) *J. Bone Miner. Res.* **12,** 165–171
13. Varghese, R., Gagliardi, A., Bialek, P., Yee, S., Wagner, G., and Dimattia, G. (2002) *Endocrinology* **143,** 868–876
14. Filvaroff, E. H., Guillet, S., Zlot, C., Bao, M., Ingle, G., Steinmetz, H., Hoeffel, J., Bunting, S., Ross, J., Carano, R. A., Powell-Braxton, L., Wagner, G. F., Eckert, R., Gerritsen, M. E., and French, D. M. (2002) *Endocrinology* **143,** 3681–3690
15. Paciga, M., Watson, A. J., DiMattia, G., and Wagner, G. (2002) *Endocrinology* **143,** 3925–3934
16. Taylor, J. M., Mack, C. P., Nolan, K., Regan, C. P., Owens, G. K., and Parsons, J. T. (2001) *Mol. Cell. Biol.* **21,** 1565–1572
17. Richardson, A., and Parsons, T. (1996) *Nature* **380,** 538–540
18. Richardson, A., Malik, R. K., Hildebrand, J. D., and Parsons, J. T. (1997) *Mol. Cell. Biol.* **17,** 6906–6914
19. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995) *Nature* **377,** 539–544
20. Lai, J. F., Kao, S. C., Jiang, S. T., Tang, M. J., Chan, P. C., and Chen, H. C. (2000) *J. Biol. Chem.* **275,** 7474–7480
21. Cobb, B. S., Schaller, M. D., Leu, T. H., and Parsons, J. T. (1994) *Mol. Cell. Biol.* **14,** 147–155
22. Chen, H. C., and Guan, J. L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 10148–10152
23. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) *Nature* **372,** 786–791
24. Polte, T. R., and Hanks, S. K. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10678–10682
25. Morishita, R., Aoki, M., Yo, Y., and Ogihara, I. (2002) *Endocr. J.* **29,** 273–284
26. Bussolino, F., Di Renzo, M. F., Ziche, M., Bocchietto, E., Olivero, M., Naldini, L., Gaudino, G., Tamagnone, L., Coffer, A., and Comoglio, P. M. (1992) *J. Cell Biol.* **119,** 629–641
27. Hayashi, S., Morishita, R., Nakamura, S., Yamamoto, K., Moriguchi, A., Nagano, T., Taiji, M., Noguchi, H., Matsumoto, K., Nakamura, T., Higaki, J., and Ogihara, T. (1999) *Circulation* **100,** Suppl. 19, II301–II308
28. Jennische, E., Ekberg, S., and Matejka, G. L. (1993) *Am. J. Physiol.* **265,** 1 Pt 1, C122–C128
29. Schmidt, N. O., Westphal, M., Hagel, C., Ergun, S., Stavrou, D., Rosen, E. M., and Lamszus, K. (1999) *Int. J. Cancer* **84,** 10–18
30. To, C. T., and Tsao, M. S. (1998) *Oncol. Rep.* **5,** 1013–1024
31. McCudden, C., James, K., Hasilo, C., and Wagner, G. (2002) *J. Biol. Chem.* **277,** 45249–45258

LEXSEE


Caution
As of: Aug 19, 2008

In re JACK R. WANDS, VINCENT R. ZURAWSKI, JR., and HUBERT J. P. SCHOEMAKER

No. 87-1454

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

858 F.2d 731; 1988 U.S. App. LEXIS 13208; 8 U.S.P.Q.2D (BNA) 1400

September 30, 1988, Decided

SUBSEQUENT HISTORY: [**1] As Amended October 20, 1988.

PRIOR HISTORY: Appealed from: Patent and Trademark Office, Board of Patent Appeals and Interferences.

CASE SUMMARY:

PROCEDURAL POSTURE: Appellant sought review of the decision of the Patent and Trademark Office Board of Patent Appeals and Interferences affirming rejection of appellant's application for a patent under 35 U.S.C.S. § 112 because appellant's written specifications would not enable a person to practice the claimed invention without undue experimentation.

OVERVIEW: Appellants contended that their written specifications fully enabled the practice of their claimed invention in accordance with 35 U.S.C.S. § 112 because the antibodies needed to perform the immunoassays could be made from readily available starting materials using methods that were well known in the antibody art. Respondent alleged that appellant's data presented that the production of antibodies was unpredictable and unreliable and that it would require undue experimentation for one skilled in the art to make the antibodies. The court agreed with appellant, holding that respondent's interpretation of the data was erroneous. Appellant's written disclosure fully enabled the claimed invention. Respondent's classification of the stored cell lines as failures was strained and unduly harsh. Appellant's explanation of its initial failures was reasonable and in view of the fact that the following six fusions were successful, the court concluded that appellant effectively rebutted respondent's challenge to 35 U.S.C.S. § 112.

OUTCOME: The court reversed the decision affirming rejection of appellant's application, concluding that appellant's written specification would not require undue experimentation to obtain antibodies needed to practice the claimed invention in order to meet the enablement requirement.

LexisNexis(R) Headnotes

Patent Law > Claims & Specifications > Definiteness > General Overview
Patent Law > Claims & Specifications > Enablement Requirement > Standards & Tests
[HN1]The first paragraph of 35 U.S.C.S § 112 requires that the specification of a patent must enable a person skilled in the art to make and use the claimed invention. Patents are written to enable those skilled in the art to practice the invention.

Patent Law > Claims & Specifications > Description Requirement > General Overview
Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN2]A patent need not disclose what is well known in the art.

Administrative Law > Judicial Review > Standards of Review > Clearly Erroneous Review
Civil Procedure > Appeals > Standards of Review > Clearly Erroneous Review
Patent Law > Claims & Specifications > Enablement Requirement > Standards & Tests
[HN3]Although the court reviews underlying facts found by the Patent and Trademark Office Board of Patent Appeals and Interferences under a clearly erroneous standard, it reviews enablement as a question of law.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN4]Although inventions involving microorganisms or other living cells often can be enabled by a deposit, a deposit is not always necessary to satisfy the enablement requirement. No deposit is necessary if the biological organisms can be obtained from readily available sources or derived from readily available starting materials through routine screening that does not require undue experimentation.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN5]Whether the specification in an application involving living cells is enabled without a deposit must be decided on the facts of the particular case.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN6]Enablement is not precluded by the necessity for some experimentation such as routine screening. However, experimentation needed to practice the invention must not be undue experimentation. The key word is "undue", not "experimentation."

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN7]The determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art. The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.

Patent Law > Claims & Specifications > Enablement Requirement > Proof
[HN8]Whether undue experimentation is needed is not a single, simple factual determination, but rather is a conclusion reached by weighing many factual considerations.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN9]Factors to be considered in determining whether a disclosure would require undue experimentation include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.

COUNSEL: Jorge A. Goldstein, of Saidman, Sterne, Kessler & Goldstein, of Washington, District of Columbia, argued for Appellant. With him on the brief was Henry N. Wixon.

John H. Raubitschek, Associate Solicitor, Commissioner of Patents and Trademarks, of Arlington, Virginia, argued for Appellee. With him on the brief were Joseph F. Nakamura, Solicitor and Fred E. McKelvey, Deputy Solicitor.

JUDGES: Smith, Newman, and Bissell, Circuit Judges. Newman, Circuit Judge, concurring in part, dissenting in part.

OPINION BY: SMITH

OPINION

[*733] SMITH, Circuit Judge.

This appeal is from the decision of the Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences (board) affirming the rejection of all remaining claims in appellant's application for a patent, serial No. 188,735, entitled "Immunoassay Utilizing Monoclonal High Affinity IgM Antibodies," which was filed September 19, 1980. [1] The rejection under 35 U.S.C. § 112, first paragraph, is based on the grounds that appellant's written specification would not enable [**2] a person skilled in the art to make the monoclonal antibodies that are needed to practice the claimed invention without undue experimentation. We reverse.

1 *In re Wands*, Appeal No. 673-76 (Bd. Pat. App. & Int. Dec. 30, 1986).

I. *Issue*

The only issue on appeal is whether the board erred, as a matter of law, by sustaining the examiner's rejection for lack of enablement under 35 U.S.C. § 112, first paragraph, of all remaining claims in appellants' patent application, serial No. 188,735.

II. *Background*

A. *The Art.*

The claimed invention involves immunoassay methods for the detection of hepatitis B surface antigen by using high-affinity monoclonal antibodies of the IgM isotype. *Antibodies* are a class of proteins (immunoglobulins) that help defend the body against invaders such as viruses and bacteria. An antibody has the potential to bind tightly to another molecule, which molecule is called an *antigen*. The body has the ability to make millions of [**3] different antibodies that bind to different antigens. However, it is only after exposure to an antigen that a complicated *immune response* leads to the production of antibodies against that antigen. For example, on the surface of hepatitis B virus particles there is a large protein called *hepatitis B surface antigen* (HBsAg). As its name implies, it is capable of serving as an antigen. During a hepatitis B infection (or when purified HBsAg is injected experimentally), the body begins to make antibodies that bind tightly and specifically to HBsAg. Such antibodies can be used as reagents for sensitive diagnostic tests (*e.g.*, to detect hepatitis B virus in blood and other tissues, a purpose of the claimed invention). A method for detecting or measuring antigens by using antibodies as reagents is called an *immunoassay*.

Normally, many different antibodies are produced against each antigen. One reason for this diversity is that different antibodies are produced that bind to different regions (determinants) of a large antigen molecule such as HBsAg. In addition, different antibodies may be produced that bind to the same determinant. These usually differ in the tightness with [**4] which they bind to the determinant. *Affinity* is a quantitative measure of the strength of antibody-antigen binding. Usually an antibody with a higher affinity for an antigen will be more useful for immunological diagnostic tests than one with a lower affinity. Another source of heterogeneity is that there are several immunoglobulin classes or *isotypes*. Immunoglobulin G (IgG) is the most common isotype in serum. Another isotype, immunoglobulin M (IgM), is prominent early in the immune response. IgM molecules are larger than IgG molecules, and have 10 antigen-binding sites instead of the 2 that are present in IgG. Most immunoassay methods use IgG, but the claimed invention uses only IgM antibodies.

For commercial applications there are many disadvantages to using antibodies from serum. Serum contains a complex mixture of antibodies against the antigen of interest within a much larger pool of antibodies directed at other antigens. These are available only in a limited supply that ends when the donor dies. The goal of monoclonal antibody technology is to produce an unlimited supply of a single purified antibody.

The blood cells that make antibodies are *lymphocytes*. Each [**5] lymphocyte makes only one kind of antibody. During an immune response, lymphocytes exposed to [*734] their particular antigen divide and mature. Each produces a *clone* of identical daughter cells, all of which secrete the same antibody. Clones of lymphocytes, all derived from a single lymphocyte, could provide a source of a single homogeneous antibody. However, lymphocytes do not survive for long outside of the body in cell culture.

Hybridoma technology provides a way to obtain large numbers of cells that all produce the same antibody. This method takes advantage of the properties of *myeloma* cells derived from a tumor of the immune system. The cancerous myeloma cells can divide indefinitely in vitro. They also have the potential ability to secrete antibodies. By appropriate experimental manipulations, a myeloma cell can be made to fuse with a lymphocyte to produce a single hybrid cell (hence, a hybridoma) that contains the genetic material of both cells. The hybridoma secretes the same antibody that was made by its parent lymphocyte, but acquires the capability of the myeloma cell to divide and grow indefinitely in cell culture. Antibodies produced by a clone of hybridoma [**6] cells (i.e., by hybridoma cells that are all progeny of a single cell) are called monoclonal antibodies. [2]

2 For a concise description of monoclonal antibodies and their use in immunoassay see *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1368-71, 231 USPQ 81, 82-83 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947, 107 S. Ct. 1606, 94 L. Ed. 2d 792 (1987).

B. *The Claimed Invention.*

The claimed invention involves methods for the immunoassay of HBsAg by using high-affinity monoclonal IgM antibodies. Jack R. Wands and Vincent R. Zurawski, Jr., two of the three coinventors of the present application, disclosed methods for producing monoclonal antibodies against HBsAg in United States patent No. 4,271,145 (the '145 patent), entitled "Process for Producing Antibodies to Hepatitis Virus and Cell Lines Therefor," which patent issued on June 2, 1981. The '145 patent is incorporated by reference into the application on appeal. The specification of the [**7]

'145 patent teaches a procedure for immunizing mice against HBsAg, and the use of lymphocytes from these mice to produce hybridomas that secrete monoclonal antibodies specific for HBsAg. The '145 patent discloses that this procedure yields both IgG and IgM antibodies with high-affinity binding to HBsAg. For the stated purpose of complying with the best mode requirement of 35 U.S.C. § 112, first paragraph, a hybridoma cell line that secretes IgM antibodies against HBsAg (the 1F8 cell line) was deposited at the American Type Culture Collection, a recognized cell depository, and became available to the public when the '145 patent issued.

The application on appeal claims methods for immunoassay of HBsAg using monoclonal antibodies such as those described in the '145 patent. Most immunoassay methods have used monoclonal antibodies of the IgG isotype. IgM antibodies were disfavored in the prior art because of their sensitivity to reducing agents and their tendency to self-aggregate and precipitate. Appellants found that their monoclonal IgM antibodies could be used for immunoassay of HbsAg with unexpectedly high sensitivity and specificity. Claims 1, 3, 7, [**8] 8, 14, and 15 are drawn to methods for the immunoassay of HBsAg using high-affinity IgM monoclonal antibodies. Claims 19 and 25-27 are for chemically modified (*e.g.*, radioactively labeled) monoclonal IgM antibodies used in the assays. The broadest method claim reads:

> 1. An immunoassay method utilizing an antibody to assay for a substance comprising hepatitis B-surface antigen (HBsAg) determinants which comprises the steps of:
>
> contacting a test sample containing said substance comprising HBsAg determinants with said antibody; and
>
> determining the presence of said substance in said sample;
>
> wherein said antibody is a monoclonal high affinity IgM antibody having a binding affinity constant for said HBsAg determinants of at least 10<9> M<-1>.

Certain claims were rejected under 35 U.S.C. § 103; these rejections have not [*735] been appealed. Remaining claims 1, 3, 7, 8, 14, 15, 19, and 25-27 were rejected under 35 U.S.C. § 112, first paragraph, on the grounds that the disclosure would not enable a person skilled in the art to make and use the invention without undue experimentation. The rejection is directed solely [**9] to whether the specification enables one skilled in the art to make the monoclonal antibodies that are needed to practice the invention. The position of the PTO is that data presented by Wands show that the production of high-affinity IgM anti-HBsAg antibodies is unpredictable and unreliable, so that it would require undue experimentation for one skilled in the art to make the antibodies.

III. *Analysis*

A. *Enablement by Deposit of Microorganisms and Cell Lines.*

[HN1]The first paragraph of 35 U.S.C. § 112 requires that the specification of a patent must enable a person skilled in the art to make and use the claimed invention. "Patents * * * are written to enable those skilled in the art to practice the invention." [3] [HN2]A patent need not disclose what is well known in the art. [4] [HN3]Although we review underlying facts found by the board under a "clearly erroneous" standard, [5] we review [**10] enablement as a question of law. [6]

> 3 *W.L. Gore & Assocs., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1556, 220 USPQ 303, 315 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851, 105 S. Ct. 172, 83 L. Ed. 2d 107 (1984).
>
> 4 *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).
>
> 5 *Coleman v. Dines*, 754 F.2d 353, 356, 224 USPQ 857, 859 (Fed. Cir. 1985).
>
> 6 *Moleculon Research Corp. v. CBS, Inc.*, 793 F.2d 1261, 1268, 229 USPQ 805, 810 (Fed. Cir. 1986), *cert. denied*, 479 U.S. 1030, 107 S. Ct. 875, 93 L. Ed. 2d 829 (1987); *Raytheon Co. v. Roper Corp.*, 724 F.2d 951, 960 n.6, 220 USPQ 592, 599 n.6 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835, 83 L. Ed. 2d 69, 105 S. Ct. 127 (1984).

Where an invention depends on the use of living materials such as microorganisms or cultured cells, it may be impossible [**11] to enable the public to make the invention (i.e., to obtain these living materials) solely by means of a written disclosure. One means that has been developed for complying with the enablement requirement is to deposit the living materials in cell depositories which will distribute samples to the public who wish to practice the invention after the patent issues. [7] Administrative guidelines and judicial decisions have clarified the conditions under which a deposit of organisms can satisfy the requirements of section 112. [8] A deposit has been held necessary for enablement where the starting materials (i.e., the living cells used to practice the invention, or cells from which the required cells can be produced) are not readily available to the

public. [9] Even when starting materials are available, a deposit has been necessary where it would require undue experimentation to make the cells of the invention from the starting materials. [10]

7 *In re Argoudelis*, 58 C.C.P.A. 769, 434 F.2d 1390, 1392-93, 168 USPQ 99, 101-02 (CCPA 1970).

8 *In re Lundak*, 773 F.2d 1216, 227 USPQ 90 (Fed. Cir. 1985); *Feldman v. Aunstrup*, 517 F.2d 1351, 186 USPQ 108 (CCPA 1975), *cert. denied*, 424 U.S. 912, 47 L. Ed. 2d 316, 96 S. Ct. 1109 (1976); Manual of Patent Examining Procedure (MPEP) 608.01(p)(C) (5th ed. 1983, rev. 1987). *See generally* Hampar, *Patenting of Recombinant DNA Technology: The Deposit Requirement*, 67 J. Pat. Trademark Off. Soc'y 569 (1985).

[**12]

9 *In re Jackson*, 217 USPQ 804, 807-08 (Bd. App. 1982) (strains of a newly discovered species of bacteria isolated from nature); *Feldman*, 517 F.2d 1351, 186 USPQ 108 (uncommon fungus isolated from nature); *In re Argoudelis*, 434 F.2d at 1392, 168 USPQ at 102 (novel strain of antibiotic-producing microorganism isolated from nature); *In re Kropp*, 143 USPQ 148, 152 (Bd. App. 1959) (newly discovered microorganism isolated from soil).

10 *In re Forman*, 230 USPQ 546, 547 (Bd. Pat. App. & Int. 1986) (genetically engineered bacteria where the specification provided insufficient information about the amount of time and effort required); *In re Lundak*, 773 F.2d 1216, 227 USPQ 90 (unique cell line produced from another cell line by mutagenesis).

In addition to satisfying the enablement requirement, deposit of organisms also can be used to establish the filing date of the application as the prima facie date of invention, [11] [*736] and to satisfy the requirement under 35 U.S.C. § 114 [**13] that the PTO be guaranteed access to the invention during pendency of the application. [12] Although a deposit may serve these purposes, we recognized, in *In re Lundak*, [13] that these purposes, nevertheless, may be met in ways other than by making a deposit.

11 *In re Lundak*, 773 F.2d at 1222, 227 USPQ at 95-96; *In re Feldman*, 517 F.2d at 1355, 186 USPQ at 113; *In re Argoudelis*, 434 F.2d at 1394-96, 168 USPQ at 103-04 (Baldwin, J. concurring).

12 *In re Lundak*, 773 F.2d at 1222, 227 USPQ at 95-96; *In re Feldman*, 517 F.2d at 1354, 186 USPQ at 112.

13 *In re Lundak*, 773 F.2d at 1222, 227 USPQ at 95-96.

A deposit also may satisfy the best mode requirement of section 112, first paragraph, and it is for this reason that the 1F8 hybridoma was deposited in connection with the '145 patent and the current application. Wands does not challenge the statements by the examiner to the effect that, [**14] although the deposited 1F8 line enables the public to perform immunoassays with antibodies produced by that single hybridoma, the deposit does not enable the generic claims that are on appeal. The examiner rejected the claims on the grounds that the written disclosure was not enabling and that the deposit was inadequate. Since we hold that the written disclosure fully enables the claimed invention, we need not reach the question of the adequacy of deposits.

B. *Undue Experimentation.*

[HN4]Although inventions involving microorganisms or other living cells often can be enabled by a deposit, [14] a deposit is not always necessary to satisfy the enablement requirement. [15] No deposit is necessary if the biological organisms can be obtained from readily available sources or derived from readily available starting materials through routine screening that does not require undue experimentation. [16] [HN5]Whether the specification in an application involving living cells (here, hybridomas) is enabled without [**15] a deposit must be decided on the facts of the particular case. [17]

14 *In re Argoudelis*, 434 F.2d at 1393, 168 USPQ at 102.

15 *Tabuchi v. Nubel*, 559 F.2d 1183, 194 USPQ 521 (CCPA 1977).

16 *Id.* at 1186-87, 194 USPQ at 525; *Merck & Co. v. Chase Chem. Co.*, 273 F. Supp. 68, 77, 155 USPQ 139, 146 (D.N.J. 1967); *Guaranty Trust Co. v. Union Solvents Corp.*, 54 F.2d 400, 403-06, 12 U.S.P.Q. (BNA) 47, 50-53 (D. Del. 1931), *aff'd*, 61 F.2d 1041, 15 U.S.P.Q. (BNA) 237 (3d Cir. 1932), *cert. denied*, 288 U.S. 614, 77 L. Ed. 987, 53 S. Ct. 405 (1933); MPEP 608.01(p)(C) ("No problem exists when the microorganisms used are known and readily available to the public.").

17 *In re Jackson*, 217 USPQ at 807; *see In re Metcalfe*, 56 C.C.P.A. 1191, 410 F.2d 1378, 1382, 161 USPQ 789, 792 (CCPA 1969).

Appellants contend that their written specification fully [**16] enables the practice of their claimed invention because the monoclonal antibodies needed to perform the immunoassays can be made from readily

available starting materials using methods that are well known in the monoclonal antibody art. Wands states that application of these methods to make high-affinity IgM anti-HBsAg antibodies requires only routine screening, and that does not amount to undue experimentation. There is no challenge to their contention that the starting materials (i.e., mice, HBsAg antigen, and myeloma cells) are available to the public. The PTO concedes that the methods used to prepare hybridomas and to screen them for high-affinity IgM antibodies against HBsAg were either well known in the monoclonal antibody art or adequately disclosed in the '145 patent and in the current application. This is consistent with this court's recognition with respect to another patent application that methods for obtaining and screening monoclonal antibodies were well known in 1980. [18] The sole issue is whether, in this particular case, it would require undue experimentation to produce high-affinity IgM monoclonal antibodies.

18 *Hybritech*, 802 F.2d at 1384, 231 USPQ at 94.

[**17] [HN6]Enablement is not precluded by the necessity for some experimentation such as [*737] routine screening. [19] However, experimentation needed to practice the invention must not be undue experimentation. [20] "The key word is 'undue,' not 'experimentation.'" [21]

> [HN7]The determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art. *Ansul Co. v. Uniroyal, Inc.* [448 F.2d 872, 878-79; 169 USPQ 759, 762-63 (2d Cir. 1971), *cert. denied*, 404 U.S. 1018, 30 L. Ed. 2d 666, 92 S. Ct. 680 (1972)]. The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed * * *. [22]

19 *Id.; Atlas Powder Co. v. E.I. DuPont De Nemours & Co.*, 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984); *In re Angstadt*, 537 F.2d at 502-504, 190 USPQ at 218; *In re Geerdes*, 491 F.2d 1260, 1265, 180 USPQ 789, 793 (CCPA 1974); *Minerals Separation, Ltd. v. Hyde*, 242 U.S. 261, 270-71, 61 L. Ed. 286, 37 S. Ct. 82 (1916).

[**18]

20 *Hybritech*, 802 F.2d at 1384, 231 USPQ at 94; *W.L. Gore*, 721 F.2d at 1557, 220 USPQ at 316; *In re Colianni*, 561 F.2d 220, 224, 195 USPQ 150, 153 (CCPA 1977) (Miller, J., concurring).

21 *In re Angstadt*, 537 F.2d at 504, 190 USPQ at 219.

22 *In re Jackson*, 217 USPQ at 807.

The term "undue experimentation" does not appear in the statute, but it is well established that enablement requires that the specification teach those in the art to make and use the invention without undue experimentation. [23] [HN8]Whether undue experimentation is needed is not a single, simple factual determination, but rather is a conclusion reached by weighing many factual considerations. The board concluded that undue experimentation would be needed to practice the invention on the basis of experimental data presented by Wands. These data are not in dispute. However, Wands and the board disagree strongly on the conclusion that [**19] should be drawn from that data.

23 *See Hybritech*, 802 F.2d at 1384, 231 USPQ at 94; *Atlas Powder*, 750 F.2d at 1576, 224 USPQ at 413.

[HN9]Factors to be considered in determining whether a disclosure would require undue experimentation have been summarized by the board in *In re Forman*. [24] They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. [25]

24 *In re Forman*, 230 USPQ at 547.

25 *Id.; see In re Colianni*, 561 F.2d at 224, 195 USPQ at 153 (Miller, J., concurring); *In re Rainer*, 52 C.C.P.A. 1593, 347 F.2d 574, 577, 146 USPQ 218, 221 (CCPA 1965).

[**20] In order to understand whether the rejection was proper, it is necessary to discuss further the methods for making specific monoclonal antibodies. The first step for making monoclonal antibodies is to immunize an animal. The '145 patent provides a detailed description of procedures for immunizing a specific strain of mice against HBsAg. Next the spleen, an organ rich in lymphocytes, is removed and the lymphocytes are separated from the other spleen cells. The lymphocytes

are mixed with myeloma cells, and the mixture is treated to cause a few of the cells to fuse with each other. Hybridoma cells that secrete the desired antibodies then must be isolated from the enormous number of other cells in the mixture. This is done through a series of screening procedures.

The first step is to separate the hybridoma cells from unfused lymphocytes and myeloma cells. The cells are cultured in a medium in which all the lymphocytes and myeloma cells die, and only the hybridoma cells survive. The next step is to isolate and clone hybridomas that make antibodies [*738] that bind to the antigen of interest. Single hybridoma cells are placed in separate chambers and are allowed to grow and divide. [**21] After there are enough cells in the clone to produce sufficient quantities of antibody to analyze, the antibody is assayed to determine whether it binds to the antigen. Generally, antibodies from many clones do not bind the antigen, and these clones are discarded. However, by screening enough clones (often hundreds at a time), hybridomas may be found that secrete antibodies against the antigen of interest.

Wands used a commercially available radioimmunoassay kit to screen clones for cells that produce antibodies directed against HBsAg. In this assay the amount of radioactivity bound gives some indication of the strength of the antibody-antigen binding, but does not yield a numerical affinity constant, which must be measured using the more laborious Scatchard analysis. In order to determine which anti-HBsAg antibodies satisfy all of the limitations of appellants' claims, the antibodies require further screening to select those which have an IgM isotype and have a binding affinity constant of at least 10<<9 M<-1>. [26] The PTO does not question that the screening techniques used by Wands were well known in the monoclonal antibody art.

> 26 The examiner, the board, and Wands all point out that, technically, the strength of antibody-HBsAg binding is measured as *avidity*, which takes into account multiple determinants on the HBsAg molecule, rather than affinity. Nevertheless, despite this correction, all parties then continued to use the term "affinity." We will use the terminology of the parties. Following the usage of the parties, we will also use the term "high-affinity" as essentially synonymous with "having a binding affinity constant of at least 10<9> M<-1>."

[**22] During prosecution Wands submitted a declaration under 37 C.F.R. § 1.132 providing information about all of the hybridomas that appellants had produced before filing the patent application. The first four fusions were unsuccessful and produced no hybridomas. The next six fusion experiments all produced hybridomas that made antibodies specific for HBsAg. Antibodies that bound at least 10,000 cpm in the commercial radioimmunoassay were classified as "high binders." Using this criterion, 143 high-binding hybridomas were obtained. In the declaration, Wands stated that [27]

> It is generally accepted in the art that, among those antibodies which are binders with 50,000 cpm or higher, there is a very high likelihood that high affinity (Ka [greater than] 10<9> M<-1>) antibodies will be found. However, high affinity antibodies can also be found among high binders of between 10,000 and 50,000, as is clearly demonstrated in the Table.

The PTO has not challenged this statement.

> 27 A table in the declaration presented the binding data for antibodies from every cell line. Values ranged from 13,867 to 125,204 cpm, and a substantial proportion of the antibodies showed binding greater than 50,000 cpm. In confirmation of Dr. Wand's statement, two antibodies with binding less than 25,000 cpm were found to have affinity constants greater than 10<9> M<-1>.

[**23] The declaration stated that a few of the high-binding monoclonal antibodies from two fusions were chosen for further screening. The remainder of the antibodies and the hybridomas that produced them were saved by freezing. Only nine antibodies were subjected to further analysis. Four (three from one fusion and one from another fusion) fell within the claims, that is, were IgM antibodies and had a binding affinity constant of at least 10<9> M<-1>. Of the remaining five antibodies, three were found to be IgG, while the other two were IgM for which the affinity constants were not measured (although both showed binding well above 50,000 cpm).

Apparently none of the frozen cell lines received any further analysis. The declaration explains that after useful high-affinity IgM monoclonal antibodies to HBsAg had been found, it was considered unnecessary to return to the stored antibodies to screen for more IgMs. Wands says that the existence of the stored hybridomas was disclosed to the PTO to comply with the requirement under 37 C.F.R. § 1.56 that applicants fully disclose all of their relevant [*739] data, and not just favorable results. [28] How these stored hybridomas are viewed [**24] is central to the positions of the parties.

28 *See Rohm & Haas Co. v. Crystal Chem. Co.*, 722 F.2d 1556, 220 USPQ 289 (Fed. Cir. 1983).

The position of the board emphasizes the fact that since the stored cell lines were not completely tested, there is no proof that any of them are IgM antibodies with a binding affinity constant of at least 10<9> M<-1>. Thus, only 4 out of 143 hybridomas, or 2.8 percent, were *proved* to fall within the claims. Furthermore, antibodies that were proved to be high-affinity IgM came from only 2 of 10 fusion experiments. These statistics are viewed by the board as evidence that appellants' methods were not predictable or reproducible. The board concludes that Wands' low rate of demonstrated success shows that a person skilled in the art would have to engage in undue experimentation in order to make antibodies that fall within the claims.

Wands views the data quite differently. Only nine hybridomas were actually analyzed beyond the initial screening for HBsAg [**25] binding. Of these, four produced antibodies that fell within the claims, a respectable 44 percent rate of success. (Furthermore, since the two additional IgM antibodies for which the affinity constants were never measured showed binding in excess of 50,000 cpm, it is likely that these also fall within the claims.) Wands argues that the remaining 134 unanalyzed, stored cell lines should not be written off as failures. Instead, if anything, they represent partial success. Each of the stored hybridomas had been shown to produce a high-binding antibody specific for HBsAg. Many of these antibodies showed binding above 50,000 cpm and are thus highly likely to have a binding affinity constant of at least 10<9> M<-1>. Extrapolating from the nine hybridomas that were screened for isotype (and from what is well known in the monoclonal antibody art about isotype frequency), it is reasonable to assume that the stored cells include some that produce IgM. Thus, if the 134 incompletely analyzed cell lines are considered at all, they provide some support (albeit without rigorous proof) to the view that hybridomas falling within the claims are not so rare that undue experimentation would be needed [**26] to make them.

The first four fusion attempts were failures, while high-binding antibodies were produced in the next six fusions. Appellants contend that the initial failures occurred because they had not yet learned to fuse cells successfully. Once they became skilled in the art, they invariably obtained numerous hybridomas that made high-binding antibodies against HBsAg and, in each fusion where they determined isotype and binding affinity they obtained hybridomas that fell within the claims.

Wands also submitted a second declaration under 37 C.F.R. § 1.132 stating that after the patent application was submitted they performed an eleventh fusion experiment and obtained another hybridoma that made a high-affinity IgM anti-HBsAg antibody. No information was provided about the number of clones screened in that experiment. The board determined that, because there was no indication as to the number of hybridomas screened, this declaration had very little value. While we agree that it would have been preferable if Wands had included this information, the declaration does show that when appellants repeated their procedures they again obtained a hybridoma that produced an antibody that [**27] fit all of the limitations of their claims.

We conclude that the board's interpretation of the data is erroneous. It is strained and unduly harsh to classify the stored cell lines (each of which was proved to make high-binding antibodies against HBsAg) as failures demonstrating that Wands' methods are unpredictable or unreliable. [29] At worst, they prove nothing at all about the probability of success, and merely show [*740] that appellants were prudent in not discarding cells that might someday prove useful. At best, they show that high-binding antibodies, the starting materials for IgM screening and Scatchard analysis, can be produced in large numbers. The PTO's position leads to the absurd conclusion that the more hybridomas an applicant makes and saves without testing, the less predictable the applicant's results become. Furthermore, Wands' explanation that the first four attempts at cell fusion failed only because they had not yet learned to perform fusions properly is reasonable in view of the fact that the next six fusions were all successful. The record indicates that cell fusion is a technique that is well known to those of ordinary skill in the monoclonal antibody [**28] art, and there has been no claim that the fusion step should be more difficult or unreliable where the antigen is HBsAg than it would be for other antigens.

29 Even if we were to accept the PTO's 2.8% success rate, we would not be required to reach a conclusion of undue experimentation. Such a determination must be made in view of the circumstances of each case and cannot be made solely by reference to a particular numerical cutoff.

When Wands' data is interpreted in a reasonable manner, analysis considering the factors enumerated in *In re Forman* leads to the conclusion that undue experimentation would not be required to practice the invention. Wands' disclosure provides considerable direction and guidance on how to practice their invention and presents working examples. There was a high level of skill in the art at the time when the application was filed, and all of the methods needed to practice the invention were well known.

The nature of monoclonal antibody technology is that it involves screening [**29] hybridomas to determine which ones secrete antibody with desired characteristics. Practitioners of this art are prepared to screen negative hybridomas in order to find one that makes the desired antibody. No evidence was presented by either party on how many hybridomas would be viewed by those in the art as requiring undue experimentation to screen. However, it seems unlikely that undue experimentation would be defined in terms of the number of hybridomas that were never screened. Furthermore, in the monoclonal antibody art it appears that an "experiment" is not simply the screening of a single hybridoma, but is rather the entire attempt to make a monoclonal antibody against a particular antigen. This process entails immunizing animals, fusing lymphocytes from the immunized animals with myeloma cells to make hybridomas, cloning the hybridomas, and screening the antibodies produced by the hybridomas for the desired characteristics. Wands carried out this entire procedure three times, and was successful each time in making at least one antibody that satisfied all of the claim limitations. Reasonably interpreted, Wands' record indicates that, in the production of high-affinity IgM antibodies [**30] against HBsAG, the amount of effort needed to obtain such antibodies is not excessive. Wands' evidence thus effectively rebuts the examiner's challenge to the enablement of their disclosure. [30]

30 *In re Strahilevitz*, 668 F.2d 1229, 1232, 212 USPQ 561, 563 (CCPA 1982).

IV. *Conclusion*

Considering all of the factors, we conclude that it would not require undue experimentation to obtain antibodies needed to practice the claimed invention. Accordingly, the rejection of Wands' claims for lack of enablement under 35 U.S.C. § 112, first paragraph, is reversed.

REVERSED

CONCUR BY: NEWMAN (In Part)

DISSENT BY: NEWMAN (In Part)

DISSENT

NEWMAN, Circuit Judge, concurring in part, dissenting in part.

A

I concur in the court's holding that additional samples of hybridoma cell lines that produce these high-affinity IgM monoclonal antibodies need not be deposited. This invention, as described by Wands, is not a selection of a few rare cells from many possible cells. To the contrary, [**31] Wands states that all monoclonally produced IgM antibodies to hepatitis B surface antigen have the desired high avidity and other favorable properties, and that all are readily preparable by now-standard techniques.

Wands states that his United States Patent No. 4,271,145 describes fully operable techniques, and is distinguished from his [*741] first four failed experiments that are referred to in the Rule 132 affidavit. Wands argues that these biotechnological mechanisms are relatively well understood and that the preparations can be routinely duplicated by those of skill in this art, as in *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1380, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947, 107 S. Ct. 1606, 94 L. Ed. 2d 792 (1987). I agree that it is not necessary that there be a deposit of multiple exemplars of a cell system that is readily reproduced by known, specifically identified techniques.

B

I would affirm the board's holding that Wands has not complied with 35 U.S.C. § 112, first paragraph, in that he has not provided data sufficient to support the breadth of his generic [**32] claims. Wands' claims on appeal include the following:

> 19. Monoclonal high affinity IgM antibodies immunoreactive with HBsAg determinants, wherein said antibodies are coupled to an insoluble solid phase, and wherein the binding affinity constant of said antibodies for said HBsAg determinants is at least 10<9> M<-1>.
>
> 26. Monoclonal high affinity IgM antibodies immunoreactive with HBsAg determinants wherein said antibodies are detectably labelled.

Wands states that he obtained 143 "high binding monoclonal antibodies of the right specificity" in the successful fusions; although he does not state how they were determined to be high binding or of the right specificity, for Wands also states that only nine of these 143 were tested.

Of these nine, four (three from one fusion and one from another fusion) were found to have the claimed high affinity and to be of the IgM isotype. Wands states that the other five were either of a different isotype or their affinities were not determined. (This latter statement also appears to contradict his statement that all 143 were "high binding".)

Wands argues that a "success rate of four out of nine", or 44.4%, is sufficient to [**33] support claims to the entire class. The Commissioner deems the success rate to be four out of 143, or 2.8%; to which Wands responds with statistical analysis as to how unlikely it is that Wands selected the only four out of 143 that worked. Wands did not, however, prove the right point. The question is whether Wands, by testing nine out of 143 (the Commissioner points out that the randomness of the sample was not established), and finding that four out of the nine had the desired properties, has provided sufficient experimental support for the breadth of the requested claims, in the context that "experiments in genetic engineering produce, at best, unpredictable results", quoting from *Ex parte Forman*, 230 USPQ 546, 547 (Bd.Pat.App. and Int. 1986).

The premise of the patent system is that an inventor, having taught the world something it didn't know, is encouraged to make the product available for public and commercial benefit, by governmental grant of the right to exclude others from practice of that which the inventor has disclosed. The boundary defining the excludable subject matter must be carefully set: it must protect the inventor, so that commercial development [**34] is encouraged; but the claims must be commensurate with the inventor's contribution. Thus the specification and claims must meet the requirements of 35 U.S.C. § 112. *In re Fisher*, 57 C.C.P.A. 1099, 427 F.2d 833, 839, 166 USPQ 18, 23-24 (CCPA 1970).

As the science of biotechnology matures the need for special accommodation, such as the deposit of cell lines or microorganisms, may diminish; but there remains the body of law and practice on the need for sufficient disclosure, including experimental data when appropriate, that reasonably support the scope of the requested claims. That law relates to the sufficiency of the description of the claimed invention, and if not satisfied by deposit, must independently meet the requirements of Section 112.

Wands is not claiming a particular, specified IgM antibody. He is claiming all such monoclonal antibodies in assay for hepatitis B surface antigen, based on his teaching that such antibodies have uniformly reproducible high avidity, free of the known [*742] disadvantages of IgM antibodies such as tendency to precipitate or aggregate. It is incumbent upon Wands to provide reasonable support for [**35] the proposed breadth of his claims. I agree with the Commissioner that four exemplars shown to have the desired properties, out of the 143, do not provide adequate support.

Wands argues that the law should not be "harsher" where routine experiments take a long time. However, what Wands is requesting is that the law be less harsh. As illustrated in extensive precedent on the question of how much experimentation is "undue", each case must be determined on its own facts. *See, e.g., W.L. Gore & Assocs., Inc. v. Garlock, Inc.* 721 F.2d 1540, 1557, 220 USPQ 303, 316 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851, 105 S. Ct. 172, 83 L. Ed. 2d 107 (1984); *In re Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 218 (CCPA 1976); *In re Cook*, 58 C.C.P.A. 1049, 439 F.2d 730, 734-35, 169 USPQ 298, 302-03 (CCPA 1971).

The various criteria to be considered in determining whether undue experimentation is required are discussed in, for example, *Fields v. Conover*, 58 C.C.P.A. 1366, 443 F.2d 1386, 170 USPQ 276 (CCPA 1971); *In re Rainer*, 52 C.C.P.A. 1593, 347 F.2d 574, 146 USPQ 218 (CCPA 1965); *Ex parte Forman*, 230 USPQ at 547. [**36] Wands must provide sufficient data or authority to show that his results are reasonably predictable within the scope of the claimed generic invention, based on experiment and/or scientific theory. In my view he has not met this burden.

IN THE MATTER OF THE APPLICATION OF HOWARD P. ANGSTADT and WILLIAM P. GRIFFIN, JR.

Patent Appeal No. 75-560

UNITED STATES COURT OF CUSTOMS AND PATENT APPEALS

537 F.2d 498; 1976 CCPA LEXIS 152; 190 U.S.P.Q. (BNA) 214

June 24, 1976, Decided.

PRIOR HISTORY: [**1] Serial No. 772,421.

CASE SUMMARY:

PROCEDURAL POSTURE: Appellants, patent applicants, sought review from a judgment of the Patent and Trademark Office Board of Appeals, affirming the rejection of certain claims in appellants' application pursuant to 35 U.S.C.S. § 112 for failure to meet patent specification requirements.

OVERVIEW: Appellants' application for a patent involving the unpredictable art of catalytic processes was rejected pursuant to 35 U.S.C.S. § 112 by the patent examiner and sustained by the Patent and Trademark Office Board of Appeals. The Board held that appellants' specification left too much conjecture, speculation, and experimentation and was insufficient in law to support the disputed claims. The court determined that the scope of the subject matter embraced by appellants' claims was clear and the claims did set out and circumscribe a particular area with a reasonable degree of precision and particularity as required by § 112. The definiteness of the language employed was analyzed in light of the teachings of the prior art and of the particular application disclosure as it would be interpreted by one possessing the ordinary level of skill in the pertinent art. Appellants' disclosure contained sufficient teaching regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and to use the claimed invention. There was no requirement to disclose every species recommended by appellants' claims even in an unpredictable art as catalytic processes.

OUTCOME: The court reversed the judgment sustaining rejection of appellants' patent application. Rejection of a certain claim as being inconsistent with another claim was also reversed.

LexisNexis(R) Headnotes

Patent Law > Claims & Specifications > Definiteness > General Overview
Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
[HN1]The first inquiry under 35 U.S.C.S. §112, is merely to determine whether the claims do, in fact, set out and circumscribe a particular area with a reasonable degree of precision and particularity. It is here where the definiteness of the language employed must be analyzed - not in a vacuum, but always in light of the teachings of the prior art and of the particular application disclosure as it would be interpreted by one possessing the ordinary level of skill in the pertinent art.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Date of Invention & Priority > General Overview
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals

[HN2]Once having determined that the subject matter defined by the claims is particular and definite, the should be an analysis of the first paragraph of 35 U.S.C.S. §112, to determine whether the scope of protection sought is supported and justified by the specification disclosure. It should be evident that these inquiries include determining whether the subject matter defined in the claims is described in the specification, whether the specification disclosure as a whole is such as to enable one skilled in the art to make and use the claimed invention, and whether the best mode contemplated by the inventor of carrying out that invention is set forth.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
[HN3]What is of maximum concern in any analysis of whether a particular claim is supported by the disclosure in an application is whether that disclosure contains sufficient teaching regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and to use the claimed invention. The relevant inquiry may be summed up as being whether the scope of enablement provided to one of ordinary skill in the art by the disclosure is such as to be commensurate with the scope of protection sought by the claims.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
[HN4]Many chemical processes, and catalytic processes particularly, are unpredictable, and the scope of enablement varies inversely with the degree of unpredictability involved.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
Patent Law > Utility Requirement > Chemical Compounds
[HN5]To require disclosures in patent applications to transcend the level of knowledge of those skilled in the art would stifle the disclosure of inventions in fields man understands imperfectly, like catalytic chemistry.

Patent Law > Inequitable Conduct > General Overview
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
Patent Law > Ownership > Conveyances > General Overview
[HN6]The Patent and Trademark Office has the burden of giving reasons, supported by the record as a whole, why the specification is not enabling. Showing that the disclosure entails undue experimentation is part of the Patent and Trademark Office's initial burden.

OPINION BY: BALDWIN

OPINION

[*499] BALDWIN, Judge.

This appeal is from the decision of the Patent and Trademark Office Board of Appeals affirming the rejection of claims 3-5, 12-22, and 27 in application serial No. 772,421, filed October 31, 1968, for "Organometallic Complexes As Alkylaromatic Oxidation Catalysts." We reverse.

The Invention

The claimed invention involves a method of catalytically oxidizing secondary or tertiary alkylaromatic hydrocarbons to form a reaction mixture comprising the corresponding hydroperoxides. The method employs as the catalyst an organometallic complex formed between hexaalkylphosphoramides and metal salts, the complex having the formula MX(n)(HAPA)(m), wherein HAPA is a hexaalkylphosphoramide, MX is a metal salt, m is an integer of from 1 to 8, and n is an integer of from 1 to 4.

In summarizing their invention, appellants state in their specification:

[*500] It has now been found, in accordance with the present invention, that organometallic complexes formed between metal salts, preferably those derived from transition metals, (including metals of the lanthanide and actinide series), and hexaalkylphosphoramides are [**2] effective catalysts in the oxidation of secondary and tertiary alkylaromatic hydrocarbons. Certain of these catalysts, and particularly those derived from metal salts of the lanthanide series, are especially effective in forming the hydroperoxides of the alkylaromtic [sic] hydrocarbons to the exclusion of other hydrocarbon oxidation products, thus providing the hydroperoxides in high yields at increased conversion rates.

The products produced by this process are described in appellants' specification as follows:

[The] oxidation products of the instant process are generally, alcohols, aldehydes, ketones, hydroperoxides, or mixtures thereof. Of these various products, maximization of the formation of the hydroperoxides is generally preferred * * *. Accordingly, as a preferred embodiment of this invention, it has been discovered that certain of the various metals * * * are particularly effective as catalysts in the preparation of hydroperoxides from secondary and tertiary alkylaromatic compounds * * *. These metals preferentially give yields of over 90 percent hydroperoxides to the exclusion of other oxidation products at conversion rates of at least about 4 percent per hour, [**3] and in many cases as high as 20 to 25 percent per hour. In the case of those remaining metals which yield lesser amounts or no hydroperoxides in the final product, while applicants do not wish to be bound by any particular theory, it is believed that they too yield hydroperoxides which are then rapidly decomposed by the catalyst complex to form aldehydes, ketones and the like. [Emphasis added.]

Claims 22 and 27 are illustrative and read as follows:

22. The process according to Claim 27 wherein the oxidation is carried out in the added presence of a hydroperoxide.

27. In the process for the catalytic oxidation of secondary or tertiary alkylaromatic hydrocarbons of the formula

[Graphic omitted. See illustration in original.]

wherein R is lower alkyl; R(1) is lower alkyl or hydrogen; and Ar is an aromatic nucleus selected from the group consisting of phenyl and naphthyl, in the presence of air or oxygen at a temperature of from about 80 to 150 degrees C to form a reaction mixture comprising the corresponding hydroperoxides, the improvement wherein the catalyst is of the formula

[Graphic omitted. See illustration in original.]

wherein HAPA is a hexaalkylphosphoramide, [**4] the alkyl moiety of which has from one to thirty carbon atoms; MX is metal salt wherein M is a transition metal cation of Group IB, IIB, IIIB, IVB, VB, VIB, VIIB, VIIIB or IIA of the Periodic Table and X is an inorganic anion of said metal salt; m is an integer of from 1 to 8; and n is an integer of from 1 to 4, wherein the ratio of said catalyst to said alkylaromatic hydrocarbon is from about 0.1 to 5.0 parts by weight of catalyst per 100 parts by weight of alkylaromatic hydrocarbon.

The Rejection

The examiner rejected all of the claims under 35 USC 112, first and second paragraphs. However, the board's rationale for affirming the rejection was directed primarily to the enablement requirement of the first paragraph of section 112. The board considered the question of "whether the claims on appeal read on subject matter as to which the specification is not enabling." The claims and the specification were compared and it was [*501] found:

[The] claims call for the preparation of a reaction mixture comprising the hydroperoxides, using the metal salt complexes as the catalyst. However, the specification states that not all of the complexes will produce hydroperoxides and [**5] neither discloses which of the complexes will not work nor gives any information as to how the operative catalysts might be determined, without undue experimentation. We believe that the specification leaves too much to conjecture, speculation and experimentation and is insufficient in law to support the present claims containing the disputed language. [Emphasis in original.]

In addition, the board specifically pointed to claim 22 as being "inconsistent with claim 27 in light of present Example 6, which indicates that the presence of added hydroperoxide results in a product devoid of the recited 'corresponding hydroperoxides.'" The board went on to state that "insofar as this is a further ground of rejection under 35 USC 112, it is sustained."

OPINION

Since all of the claims have been rejected under the first and second paragraphs of 35 USC 112, we begin with the analysis set forth in In re Moore, 58 CCPA 1042, 1046-47, 439 F.2d 1232, 1235, 169 USPQ 236, 238 (1971):

Any analysis in this regard should begin with the determination of whether the claims satisfy the requirements of the second paragraph. It may appear awkward at first to consider the two paragraphs in inverse [**6] order but it should be realized that when the first paragraph speaks of "the invention", it can only be referring to that invention which the applicant wishes to have protected by the patent grant, i.e., the claimed invention. For this reason the claims must be analyzed first in order to determine exactly what subject matter they encompass. The subject matter there set out must be presumed, in the absence of evidence to the contrary, to be that "which the applicant regards as his invention."

[HN1]This first inquiry therefore is merely to determine whether the claims do, in fact, set out and circumscribe a particular area with a reasonable degree of precision and particularity. It is here where the definiteness of the language employed must be analyzed - not in a vacuum, but always in light of the teachings of the prior art and of the particular application disclosure

as it would be interpreted by one possessing the ordinary level of skill in the pertinent art. [Footnote omitted, original emphasis.]

We note at the outset that the claim limitation "to form * * * hydroperoxides" must be given effect since we must give effect to all claim limitations. See In re Geerdes, 491 F.2d [**7] 1260, 180 USPQ 789 (CCPA 1974); In re Wilder, 57 CCPA 1314, 429 F.2d 447, 166 USPQ 545 (1970). Furthermore, the use of functional language is sanctioned specifically by the third paragraph of section 112. Finally, in this particular case, the functional limitation was inserted in the claims at the specific insistence of the examiner.

We further note that, while the board affirmed the examiner's rejection of the claims under both the first and second paragraphs of section 112, it stated that the primary issue is "whether the claims on appeal read on subject matter as to which the specification is not enabling." This is a section 112, first paragraph issue. In re Geerdes, supra. At any rate, we conclude that the scope of the subject matter embraced by the instant claims is clear and the claims do, in fact, set out and circumscribe a particular area with a reasonable degree of precision and particularity. In re Moore, supra at 1046-47, 439 F.2d at 1235, 169 USPQ at 238. Therefore, the rejection under the second paragraph of 35 USC 112 is reversed.

Continuing with the analysis described in Moore, supra:

[HN2]Once having determined that the subject matter defined by the claims is particular [**8] and definite, the analysis then [*502] turns to the first paragraph of section 112 to determine whether the scope of protection sought is supported and justified by the specification disclosure. This first paragraph analysis in itself contains several inquiries. Considering the language of the statute, it should be evident that these inquiries include determining whether the subject matter defined in the claims is described in the specification, whether the specification disclosure as a whole is such as to enable one skilled in the art to make and use the claimed invention, and whether the best mode contemplated by the inventor of carrying out that invention is set forth.

Two of the first paragraph requirements indicated above, i.e., the "description of the invention" and the "best mode" requirements, are relatively simple to comply with and thus will ordinarily demand minimal concern on the part of the Patent Office. * * * [HN3]What is of maximum concern in any analysis of whether a particular claim is supported by the disclosure in an application is whether that disclosure contains sufficient teaching regarding the subject matter of the claims as to enable one skilled in the [**9] pertinent art to make and to use the claimed invention. These two requirements, "how to make" and "how to use" have sometimes been referred to in combination as the "enablement" requirement, but, in one form or another, have been the subject of extended discussion in this court of recent years. The relevant inquiry may be summed up as being whether the scope of enablement provided to one of ordinary skill in the art by the disclosure is such as to be commensurate with the scope of protection sought by the claims. [58 CCPA at 1047, 439 F.2d at 1235-36, 169 USPQ at 238-39, original emphasis.]

We cannot agree with the board that appellants' disclosure is not sufficient to enable one of ordinary skill in the art to practice the invention without undue experimentation. We note that [HN4]many chemical processes, and catalytic processes particularly, are unpredictable, In re Mercier, 515 F.2d 1161, 1167-68, 185 USPQ 774, 779 (CCPA 1975), and that the scope of enablement varies inversely with the degree of unpredictability involved, In re Fisher, 57 CCPA 1099, 1108, 427 F.2d 833, 839, 166 USPQ 18, 24 (1970). That this particular process is unpredictable is demonstrated further by appellants [**10] in their specification. Appellants have disclosed forty examples; one of these examples yields no hydroperoxides in the final product. Also, appellants have expressly indicated in their specification that some of these organometallic complex catalysts "yield * * * no hydroperoxides in the final product." [1]

> 1 The unpredictability of appellants' process is also evidenced by the fact that nickel (run 1, Table I), cupric (run 2, Table I), and zinc nickel (run 1, Table I), cupric well as silver nitrate (run 8, Table I) are useful (i.e., produce hydroperoxides) in appellants' process whereas cuprous chloride (run 15, Table I) is not. (Nickel and zinc are directly to the left and right respectively of copper in the periodic table; silver is directly below copper in the periodic table.)

Appellants have apparently not disclosed every catalyst which will work; they have apparently not disclosed every catalyst which will not work. The question, then, is whether in an unpredictable art, section 112 requires disclosure of a test with every species covered by a claim. To require such a complete disclosure would apparently necessitate a patent application or applications with "thousands" [**11] [2] of examples or the disclosure of "thousands" of catalysts along with information as to whether each exhibits catalytic behavior resulting in the production of hydroperoxides. More importantly, such a requirement would force an inventor seeking adequate patent

protection to carry out a prohibitive number of [*503] actual experiments. This would tend to discourage inventors from filing patent applications in an unpredictable area since the patent claims would have to be limited to those embodiments which are expressly disclosed. A potential infringer could readily avoid "literal" infringement of such claims by merely finding another analogous catalyst complex which could be used in "forming hydroperoxides."

> 2 The solicitor, without refutation by appellants, states: "Claim 27 literally reads on thousands of metal salt complexes in which the metal salt moiety may comprise any one of at least 50 metal cations combined with any inorganic anion."

Having decided that appellants are not required to disclose every species encompassed by their claims even in an unpredictable art such as the present record presents, each case must be determined on its own facts. In the instant [**12] case, appellants' invention is the use of a complex catalyst comprising a hexaalkylphosphoramide and a transition metal salt to catalyze the oxidation of secondary or tertiary alkylaromatic hydrocarbons to form hydroperoxides. Appellants have, in effect, provided those skilled in this art with a large but finite list of transition metal salts from which to choose in preparing such a complex catalyst. Appellants have actually carried out 40 runs using various transition metal salts and hexaalkylphosphoramides. If one skilled in this art wished to make and use a transition metal salt other than those disclosed in appellants' 40 runs, he would merely read appellants' specification for directions how to make and use the catalyst complex to oxidize the alkylaromatic hydrocarbons, and could then determine whether hydroperoxides are, in fact, formed. The process discovered by appellants is not complicated, and there is no indication that special equipment or unusual reaction conditions must be provided when practicing the invention. One skilled in this art would merely have to substitute the correct mass of a transition metal salt for the transition metal salts disclosed in appellants' [**13] 40 runs. Thus, we have no basis for concluding that persons skilled in this art, armed with the specification and its 40 working examples, would not easily be able to determine which catalyst complexes within the scope of the claims work to produce hydroperoxides and which do not.

Since appellants have supplied the list of catalysts and have taught how to make and how to use them, we believe that the experimentation required to determine which catalysts will produce hydroperoxides would not be undue and certainly would not "require ingenuity beyond that to be expected of one of ordinary skill in the art." Fields v. Conover, 58 CCPA 1366, 1372, 443 F.2d 1386, 1390-91, 170 USPQ 276, 279 (1971).

The dissent's reliance on In re Rainer, 54 CCPA 1445, 377 F.2d 1006, 153 USPQ 802 (1967), is misplaced. If Rainer stands for the proposition that the disclosure must provide "guidance which will enable one skilled in the art to determine, with reasonable certainty before performing the reaction, whether the claimed product will be obtained" (emphasis in original), as the dissent claims, then all "experimentation" is "undue," since the term "experimentation" implies that the success of the [**14] particular activity is uncertain. Such a proposition is contrary to the basic policy of the Patent Act, which is to encourage disclosure of inventions and thereby to promote progress in the useful arts. [HN5]To require disclosures in patent applications to transcend the level of knowledge of those skilled in the art would stifle the disclosure of inventions in fields man understands imperfectly, like catalytic chemistry. The Supreme Court said it aptly in Minerals Separation, Ltd. v. Hyde, 242 U.S. 261, 270-71 (1916), in discussing the adequacy of the disclosure of the froth flotation process of ore separation:

> Equally untenable is the claim that the patent is invalid for the reason that the evidence shows that when different ores are treated preliminary tests must be made to determine the amount of oil and the extent of agitation necessary in order to obtain the best results. Such variation of treatment must be within the scope of the claims, and the certainty which the law requires in patents is not greater than is reasonable, having regard to their subject-matter. The composition of ores varies infinitely, each one presenting its special problem, and it is obviously impossible [**15] to specify in a patent the precise [*504] treatment which would be most successful and economical in each case. The process is one for dealing with a large class of substances and the range of treatment within the terms of the claims, while leaving something to the skill of persons applying the invention, is clearly sufficiently definite to guide those skilled in the art to its successful application, as the evidence abundantly shows. This satisfies the law. Mowry v. Whitney, 14 Wall. 620; Ives v. Hamilton, 92 U.S. 426, and Carnegie Steel Co. v. Cambria Iron Co., 185 U.S. 403, 436, 437 [Emphasis added.]

Appellants have broadly disclosed a class of catalyst complexes whose use they deem to be part of their invention. But for this disclosure the public may have been deprived of the knowledge of appellants' process. In this art the performance of trial runs using different catalysts is "reasonable," even if the end result is uncertain, and we see no reason on this record why appellants should not be able to claim as their invention the broad range of processes which they have discovered.

The PTO withdrew a rejection of the claims under 35 USC 103, and it appears that persons [**16] skilled in this art would know how to perform processes within the scope of the claims, within the ambit of the types and amount of experimentation which the uncertainty of this art makes inevitable.

The kind of "guidance" which the dissent seems to contemplate is unrealistic in view of the nature of the invention. The examples, both operative and inoperative, are the best guidance this art permits, as far as we can conclude from the record.

We note that [HN6]the PTO has the burden of giving reasons, supported by the record as a whole, why the specification is not enabling. In re Armbruster, 512 F.2d 676, 185 USPQ 152 (CCPA 1975). Showing that the disclosure entails undue experimentation is part of the PTO's initial burden under Armbruster; this court has never held that evidence of the necessity for any experimentation, however slight, is sufficient to require the applicant to prove that the type and amount of experimentation needed is not undue.

By calling the claimed "invention" the "scope of protection sought" the dissent obscures the problem and frustrates the intended operation of the patent system. Depriving inventors of claims which adequately protect them and limiting [**17] them to claims which practically invite appropriation of the invention while avoiding infringement inevitably has the effect of suppressing disclosure. What the dissent seems to be obsessed with is the thought of catalysts which won't work to produce the intended result. Appellants have enabled those in the art to see that this is a real possibility, which is commendable frankness in a disclosure. Without undue experimentation or effort or expense the combinations which do not work will readily be discovered and, of course, nobody will use them and the claims do not cover them. The dissent wants appellants to make everything predictable in advance, which is impracticable and unreasonable.

We hold that the evidence as a whole, including the inoperative as well as the operative examples, negates the PTO position that persons of ordinary skill in this art, given its unpredictability, must engage in undue experimentation to determine which complexes work. The key word is "undue," not "experimentation." See Fields v. Conover, supra.

The rejection of claim 22 as being inconsistent with claim 27 in light of Example 6 is also reversed. First, it is not clear on this record whether [**18] the examiner actually made a separate rejection of claim 22 on this ground. If the examiner did not make such a rejection, then appellants could not acquiesce in the rejection as the board concluded.

Second, there is no reason to believe that the product recited in Example 6 is, in fact, devoid of hydroperoxides. Run 7 (Table I) and the fourth run in Example 8 (Table VI) both use the exact same metal salt, albeit a [*505] different alkylaromatic hydrocarbon starting material, as in Example 6 and hydroperoxides are produced in both cases. Example 6 merely states that the white solid material produced in the process "was identified as actaldehyde [sic]." Example 6 clearly does not expressly state that no hydroperoxides were produced. Nor does it implicitly so state in view of the well-known fact that products of organic chemical reactions are almost never produced in a 100% yield but are generally contaminated with other side products. We believe that one of those side products may be a hydroperoxide in view of the fact that hydroperoxides were produced in other runs involving the same metal salt catalyst. [3] Appellants may not have believed it necessary to state that [**19] hydroperoxides were, in fact, a (minor) product of Example 6 since the claims as originally filed did not require that appellants' process "form hydroperoxides." [4]

3 We note also that Example 4 employs the same reactant (secbutylnaphthalene) as used in Example 6 but different metal salt catalysts (NdCl(3) HMPA and TiCl(4) HMPA) and hydroperoxides are produced. (HMPA stands for "hexamethylphosphoramide").

4 Claim 1, as originally filed, reads as follows:

> A process for the oxidation of secondary and tertiary alkylaromatic compounds which comprises contacting said compounds with oxygen at an elevated temperature in the presence of a catalyst comprising a hexaalkylphosphoramide and a metal salt.

Finally, there is no record evidence to establish that the presence of hydroperoxides in Example 6 results in a product which is devoid of the recited "corresponding hydroperoxides." Because of this lack of record evidence, we discern no inconsistency between claims 22 and 27 in light of Example 6. Accordingly, the decision of the board is reversed.

REVERSED

DISSENT BY: MILLER

DISSENT

MILLER, Judge, dissenting.

The controlling issue in this case is: Whether the scope of enablement [**20] (represented by the processes disclosed in the specification) is commensurate with the scope of protection sought by the claims, which

include the limitation "to form a reaction mixture comprising the corresponding hydroperoxides."

Although it is evident that by following some of appellants' examples a reaction mixture will be formed comprising the corresponding hydroperoxides, it does not follow that the scope of enablement is commensurate with the scope of the claims. As this court well said in In re Rainer, 52 CCPA 1593, 1597, 347 F.2d 574, 578, 146 USPQ 218, 221 (1965), discussing an inadequate disclosure:

[This] failure stems not from the absence of working examples but rather from the failure of appellants to disclose any factors which would cause a person of skill in this art to select from the 53 listed materials those which will produce the claimed product.

Indeed, if this court permits, as would the majority, the number of examples to decide the question of enablement, regardless of the breadth of the claims and the amount of experimentation required by the absence of guidance in the disclosure, an applicant for a patent in an unpredictable art can assure success [**21] of his application simply by playing the numbers game. The majority has given no reason for ignoring this court's policy that "there is no magical relation between the number of representative examples and the breadth of the claims" with respect to enablement. In re Borkowski, 57 CCPA 946, 952-53, 422 F.2d 904, 910, 164 USPQ 642, 646 (1970).

The later Rainer case (54 CCPA 1445, 377 F.2d 1006, 153 USPQ 802 (1967)), well illustrates appellants' problem here. There the main claim in issue under an inadequate disclosure rejection recited a bottle made of an "irradiated polyethylene ... having grafted thereto a polymer formed by polymerizing a member of the group" (of selected monomers). The specification recited various suitable monomers. As in the present case, preference was stated for one subgroup. This consisted of [*506] hydrocarbon monomers. A second subgroup was also disclosed:

Also, there can be used monomers such as alkyl acrylates and methacrylates ..., N,N-methylene-bis-acrylamide, polyallyl esters ..., dialkenyl oxalates ..., triallyl melamine, dialkyl maleates and fumarates ....

The specification included numerous examples of bottles formed by a graft polymerization [**22] of polyethylene with various monomers. It also contained an example of a specific monomer from the second subgroup which would not graft polyethylene. No guidance was provided concerning the reactants within the scope of the claim which formed the claimed product. The court's opinion quotes from the Solicitor's explanation of the rationale of the board's rejection:

Essentially, it is based on the fact that, although the present specification catalogs a large number of monomers * * * which can be irradiated with polyethylene to form graft cross-linked copolymers, that catalog of monomers includes ethylene glycol dimethacrylate which further on in the specification * * * is shown not to form a graft cross-linked copolymer * * *. Consequently, the Board properly concluded that the disclosure would be considered adequate as to those monomers actually shown by the examples (or otherwise in the original disclosure) to form graft cross-linked copolymers with the polyethylene * * * but inadequate as to any other monomers or the very broad category of "polymerizable ethylenically unsaturated hydrocarbon monomers" ....

In affirming the board, this court said:

The thrust of the inadequate [**23] disclosure rejection is that appellants have claimed more broadly than they have invented. It is maneuvered into the shadow, at least, of section 112 by the observation that the specification gives no indication whether these monomers for which there is no specific example will behave on irradiation like styrene, which forms a graft copolymer, or like ethylene glycol dimethacrylate, which does not.

Here the board has pointed to appellants' own specification, which concedes that some organometallic complex catalysts "yield ... no hydroperoxides in the final product." (Emphasis added.) Moreover, the Solicitor has pointed to examples in appellants' specification where no hydroperoxide was formed in the final product. Thus, in run 15 of Example 2, using Cu(2)Cl(2) as the metal salt of the organometallic complex catalyst and cumene as the alkylaromatic hydrocarbon, no hydroperoxide was found in the final product. In Example 6, using MnCl(2) as the metal salt of the organometallic complex catalyst and sec - butylnaphthalene as the alkylaromatic hydrocarbon, the final product did not contain hydroperoxide. [1] Since the organometallic complex catalysts and the alkylaromatic hydrocarbons [**24] used in these two examples fall within the materials recited in the claims, and since appellants' specification states that some catalysts will not produce hydroperoxide in the final product, [2] the burden of substantiating the doubts of the Patent and Trademark Office concerning the sufficiency of the disclosure has been met. [3] As this [*507] court said in the later Rainer case, supra, 54 CCPA at 1452, 377 F.2d at 1012, 153 USPQ at 807:

1 The majority states that "there is no reason to believe that the product recited in Example 6 is, in fact, devoid of hydroperoxides," noting that run 7 and the fourth run in Example 8 "both use

the exact same metal salt, albeit a different alkylaromatic hydrocarbon starting material, as in Example 6 and hydroperoxides are produced in both cases" and that "Example 4 employs the same reactant ... as used in Example 6 but different metal salt catalysts ... and hydroperoxides are produced." The reason, of course, is that a different reactant or a different metal salt catalyst was used in Example 6. Moreover, appellants in their main brief have not argued that Example 6 produced hydroperoxide and in their reply brief have not controverted the Solicitor's statement in his brief that Example 6 did not produce hydroperoxide.

2 Appellants' theory that the hydroperoxide is formed as an intermediate is unresponsive to the clear language of claim 27 that the reaction is to form a "mixture comprising the corresponding hydroperoxides."

3 The initial burden of proof was on the Patent and Trademark Office. As we stated in In re Dinh-Nguyen, 492 F.2d 856, 858, 181 USPQ 46, 47 (CCPA 1974):

> Any assertion by the Patent Office that the enabling disclosure is not commensurate in scope with the protection sought must be supported by evidence or reasoning substantiating the doubts so expressed.
>
> Contrary to the majority opinion, which provides no citation of authority, it was not necessary for the Patent and Trademark Office initially to also show that undue experimentation is required to substantiate its doubts. See In re Armbruster, 512 F.2d 676, 185 USPQ 152 (CCPA 1975).

[**25] The present case is unusual in that appellants' specification is the evidence of its own inadequacy. The board properly relied upon it.

See also Judge Baldwin's concurring opinion in In re Mayhew, 527 F.2d 1229, 188 USPQ 356 (CCPA 1976). Thus, the burden shifted to appellants to show that their teaching of producing hydroperoxides is commensurate in scope with the claims. In re Cook, 58 CCPA 1049, 439 F.2d 730, 169 USPQ 298 (1971); In re Marzocchi, 58 CCPA 1069, 439 F.2d 220, 169 USPQ 367 (1971).

The majority erroneously attempts to meet the "undue experimentation" problem by saying that if one skilled in the art wished to make and use a transition metal salt other than those disclosed in the examples, "he would merely read appellants' specification for directions how to make and use the catalyst complex ... and could then determine [by conventional techniques] whether hydroperoxides are, in fact, formed." (Emphasis added.) This approach violates the logic of the Rainer cases that there must be guidance which will enable one skilled in the art to determine, with reasonable certainty before performing the reaction, whether the claimed product will be obtained. See Minerals [**26] Separation, Ltd. v. Hyde, 242 U.S. 261, 270 (1916). The testing advocated by the majority's reference to "experimentation, however slight" simply ignores the breadth of the scope of the claims to all reactants and catalysts that produce hydroperoxides. The majority appears to confuse type of testing with degree of testing, and it is the undue degree with which we are concerned. The majority appears to suggest that there can be no undue experimentation because of the "amount of experimentation which the uncertainty of this art makes inevitable." However, this court stated in Fields v. Conover, 58 CCPA 1366, 1372, 443 F.2d 1386, 1390-91, 170 USPQ 276, 279 (1971):

> [A] disclosure complies with the how-to-make requirement of 35 USC 112 even though "some experimentation, provided it is not an undue amount" (and provided it does not require ingenuity beyond that to be expected of one of ordinary skill in the art), is still required ....

Although appellants' specification shows some 38 examples (embodiments) within the broad scope of the claims, this number is minute in comparison with the immense number of combinations of organometallic catalysts and alkylaromatic hydrocarbons within [**27] that scope. [4] The specification fails to provide guidance for determining which of the combinations are proper and which are not. For example, no guidance is shown with respect to the reactants (alkylaromatic hydrocarbons) which should be used with a manganese-containing catalyst to predictably produce hydroperoxide. The specification lists 12 metals as particularly effective in catalysts, but the claims are not limited to these 12. The 53 metals remaining from the broadly recited group are described as "those remaining metals which yield lesser amounts or no hydroperoxides in the final product." With respect to the 53, the specification states that the applicants "do not wish to be bound by any particular theory" concerning the yielding of lesser amounts or no hydroperoxides in the final product. Of the 38 examples, 26 involve metals on the list of 12. Of the [*508] remaining 53 metals, only 12 are involved in the other 12 examples; and 2 of these are involved in 2 additional examples which were unsuccessful. Thus, we have examples (embodiments) involving only 22 of the 65 metals plus 2 which involve both successful and unsuccessful results. At best, in the case of 43 [**28] metals and the thousands of combinations in which they would be included, along with varying reaction conditions, experimentation would

be required to determine operability. There is simply no teaching of how to choose those secondary and tertiary alkylaromatic hydrocarbons and organometallic catalysts which will form hydroperoxides. The need for guidance to enable the invention, with its claims to a myriad of combinations of organometallic catalysts and alkylaromatic hydrocarbons, to be practiced without undue experimentation is evident.

> 4 The Solicitor, without refutation by appellants, states: "Claim 27 literally reads on thousands of metal salt complexes in which the metal salt moiety may comprise any one of at least 50 metal cations combined with any inorganic anion."

This court has stated that in determining whether a specification is "clearly sufficiently definite to guide those skilled in the art" to practice the invention, "nothing must be left to speculation or doubt." In re Eltgroth, 57 CCPA 833, 837, 419 F.2d 918, 921, 164 USPQ 221, 223 (1970).

The court has also long recognized that catalytic phenomena and chemical reactions are unpredictable. In re Mercier, [**29] 515 F.2d 1161, 1167-68, 185 USPQ 774, 779 (CCPA 1975); In re Marzocchi, supra, 58 CCPA at 1073, 439 F.2d at 223, 169 USPQ at 369-70. [5] As stated by the court in in re Fisher, 57 CCPA 1099, 1108, 427 F.2d 833, 839, 166 USPQ 18, 24 (1970):

> 5 Appellants' argument that the catalytic oxidation process of hydrocarbons is extremely well known, as are the corresponding products formed, is not supported by evidence; and even if it were, it does not respond to the problem of unpredictability. Argument of counsel cannot take the place of evidence. In re Schulze, 52 CCPA 1422, 346 F.2d 600, 145 USPQ 716 (1965); In re Cole, 51 CCPA 919, 326 F.2d 769, 140 USPQ 230 (1964).

In cases involving predictable factors, such as mechanical or electrical elements, a single embodiment provides broad enablement in the sense that, once imagined, other embodiments can be made without difficulty and their performance characteristics predicted by resort to known scientific laws. In cases involving unpredictable factors, such as most chemical reactions and physiological activity, the scope of enablement obviously varies inversely with the degree of unpredictability of the factors involved.

Obviously [**30] this does not mean that the unpredictablility of catalysts requires a working example for each embodiment within a claim. It does mean that appellants' specification must contain guidance for choosing the proper combinations of catalysts and alkylaromatic hydrocarbons. As the board well said, "the specification leaves too much to conjecture, speculation and experimentation." Thus, if appellants' claims had been limited to the 12 metals referred to above, and the examiner had failed to sufficiently challenge enablement with evidence or reasoning, a working example for each embodiment within the claims would be unnecessary. Moreover, if appellants had broadened their claims so that the specific oxidation product was not recited and the examiner had failed to sufficiently challenge appellants' assertion that all of the specified reactants were oxidized in the presence of the specified catalysts, a working example for each embodiment within the claims would be unnecessary. Accordingly, the majority's posing of the question whether a working example is required for each embodiment within the claims is merely the use of the "straw man" technique.

Although appellants have claimed an [**31] improved method for forming a reaction mixture comprising the corresponding hydroperoxides where the products formed are not part of the invention, enablement for forming such products, even if they are well known, is nonetheless required. See In re Joliot, 47 CCPA 722, 727, 270 F.2d 954, 958, 123 USPQ 344, 347 (1959), cert. denied, 362 U.S. 977, 125 USPQ 667 (1960).

In view of the foregoing, it is clear that appellants' specification does not enable one of ordinary skill in the art to practice the invention as broadly claimed without undue [*509] experimentation. The board properly affirmed the examiner's rejection of all claims under the first paragraph of 35 USC 112.

IN RE MIGUEL F. BRANA, JOSE M. C. BERLANGA, MARINA M. MOSET, ERICH SCHLICK and GERHARD KEILHAUER

93-1393

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

51 F.3d 1560; 1995 U.S. App. LEXIS 6362; 34 U.S.P.Q.2D (BNA) 1436

March 30, 1995, Decided

SUBSEQUENT HISTORY: [**1] As Amended March 30, 1995.

PRIOR HISTORY: Appealed from: U.S. Patent and Trademark Office Board of Patent Appeals and Interferences. (Serial No. 07/533,944).

DISPOSITION: REVERSED

CASE SUMMARY:

PROCEDURAL POSTURE: Appellant applicants sought review of a United States Patent and Trademark Office Board of Patent Appeals and Interferences decision affirming a patent examiner's rejection of the applicants' claims based on a challenge to the utility of the claimed compounds and the amount of experimentation necessary to use the compounds.

OVERVIEW: The applicants filed specifications stating that the application's non-symmetrical substitutions produced compounds with better action and better action spectrum as anti-tumor substances than known drug research. The application was rejected because the specification failed to describe any specific disease against which the compounds were active and the prior art and other tests disclosed in the specification were insufficient to establish expectation that the claimed compounds had a practical utility. The court reversed, holding that the tumor models represented a specific disease against which the claimed compounds were alleged to be effective, and the applicants' specification alleged a sufficiently specific use. In addition, the court held that taking the nature of the invention, one skilled in the art would be without basis to reasonably doubt the applicants' asserted utility on its face. Even if one so skilled would have reasonably questioned the asserted utility, the applicants proffered sufficient evidence to convince one so skilled of the asserted utility.

OUTCOME: The rejection of the applicants' claims was reversed.

LexisNexis(R) Headnotes

Patent Law > Subject Matter > Products > Compositions of Matter
Patent Law > U.S. Patent & Trademark Office Proceedings > Continuation Applications > General Overview
Patent Law > Utility Requirement > Proof of Utility
[HN1]The requirement that an invention have utility is found in 35 U.S.C.S. § 101, which states that whoever invents any new and useful composition of matter may obtain a patent therefor.

Patent Law > Claims & Specifications > Definiteness > General Overview
Patent Law > Claims & Specifications > Description Requirement > General Overview
[HN2]The requirement that an invention have utility is implicit in 35 U.S.C.S. § 112 P1, which reads that the specification shall contain a written description of the invention, and of the manner and process of making and

using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Patent Law > Claims & Specifications > Claim Language > General Overview
Patent Law > Claims & Specifications > Description Requirement > General Overview
Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN3]A specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of 35 U.S.C.S. § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

Patent Law > Utility Requirement > Proof of Utility
[HN4]The United States Patent and Trademark Office has the initial burden of challenging a presumptively correct assertion of utility in the disclosure.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Inequitable Conduct > General Overview
Patent Law > Utility Requirement > Proof of Utility
[HN5]Only after the United States Patent and Trademark Office provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility.

Patent Law > Utility Requirement > Proof of Utility
[HN6]Although it is true that minor changes in chemical compounds can radically alter their effects on the human body, evidence of success in structurally similar compounds is relevant in determining whether one skilled in the art would believe an asserted utility.

Patent Law > Utility Requirement > Proof of Utility
[HN7]Proof of an alleged pharmaceutical property for a compound by statistically significant tests with standard experimental animals is sufficient to establish utility.

Patent Law > Utility Requirement > Proof of Utility
[HN8]Food and Drug Administration approval is not a prerequisite for finding a compound useful within the meaning of the patent laws.

Patent Law > Remedies > Collateral Assessments > Costs
Patent Law > Utility Requirement > Chemical Compounds
Patent Law > Utility Requirement > Proof of Utility
[HN9]Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans.

Administrative Law > Judicial Review > Standards of Review > Clearly Erroneous Review
Civil Procedure > Appeals > Standards of Review > Clearly Erroneous Review
Patent Law > U.S. Patent & Trademark Office Proceedings > General Overview
[HN10]An appellate court's standard of review is, with regard to questions of law, that review is without deference to the views of an agency, and with regard to questions of fact, an appellate court defers to an agency unless its findings are clearly erroneous.

Civil Procedure > Judgments > General Overview
Governments > Federal Government > Employees & Officials
Patent Law > Jurisdiction & Review > Standards of Review > General Overview
[HN11]With regard to judgment calls, those questions that fall somewhere near the middle of the fact-law spectrum, an appellate court has recognized the falseness of the fact-law dichotomy, since the determination at issue, involving as it does the application of a general legal standard to particular facts, is probably most realistically described as neither of fact nor law, but mixed. When these questions of judgment are before an appellate court, whether it defers to agency judgment, and the extent to which it defers, turns on the nature of the case and the nature of the judgment.

COUNSEL: Malcolm J. MacDonald, Keil & MacDonald, of Washington, D.C., argued for appellant. With him on the brief was Herbert B. Keil. Of Counsel was David S. Nagy.

Fred E. McKelvey, Solicitor, Office of the Solicitor, of Arlington, Virginia, argued for appellee. With him on the brief were Albin F. Drost, Deputy Solicitor, Richard E. Schafer, Teddy S. Gron, Joseph G. Piccolo and Richard L. Torczon, Associate Solicitors.

JUDGES: Before PLAGER, LOURIE, and RADER, Circuit Judges.

OPINION BY: PLAGER

OPINION

[*1562] PLAGER, ***Circuit Judge***.

Miguel F. Brana, ***et al.*** (applicants), appeal the March 19, 1993 decision of the United States Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences (Board), in Appeal No. 92-1196. The Board affirmed the examiner's rejection of claims 10-13 of patent application Serial No. 533,944 under 35 U.S.C. § 112 P1 (1988). [1] The examiner's rejection, upon which the Board relied in rendering its decision, was based specifically on a challenge to the utility of the claimed compounds and the amount of experimentation necessary to use the compounds. [**2] We conclude the Board erred, and reverse.

1 Unless otherwise noted, all United States Code citations are to the 1988 edition.

I. BACKGROUND

On June 30, 1988, applicants filed patent application Serial No. 213,690 (the '690 application) [2] directed to 5-nitrobenzodeisoquinoline-1,3-dione compounds, for use as antitumor substances, having the following formula:

where n is 1 or 2, R1 and R2 are identical or different and are each hydrogen, C1-C6-alkyl, C1-C6-hydroxyalkyl, pyrrolidinyl, morpholino, piperidinyl or piperacinyl, and R3 and R4 are identical or different and are each hydrogen, C1-C6-alkyl, C1-C6-acyl, C2-C7-alkoxycarbonyl, ureyl, aminocarbonyl or C2-C7-alkylaminocarbonyl. These claimed compounds differ from several prior art benzodeisoquinoline-1,3-dione compounds due to the presence of a nitro group (O2N) at the 5-position and an amino or other amino group (NR3R4) at the 8-position of the isoquinoline ring.

2 This is a divisional of patent application Serial No. 110,871 filed October 21, 1987.

[**3] The specification states that these non-symmetrical substitutions at the 5- and 8-positions produce compounds with "a better action and a better action spectrum as antitumor substances" than known benzodeisoquinolines, namely those in K.D. Paull et al., ***Computer Assisted Structure-Activity Correlations,*** **Drug Research, 34(II),** 1243-46 (1984) (Paull). Paull describes a computer-assisted evaluation of benzodeisoquinoline-1,3-diones and related compounds which have been screened for antitumor activity by testing their efficacy ***in vivo*** [3] against two specific implanted murine (i.e., utilizing mice as test subjects) lymphocytic leukemias, P388 and L1210. [4] These two ***in vivo*** tests are [*1563] widely used by the National Cancer Institute (NCI) to measure the antitumor properties of a compound. Paull noted that one compound in particular, benzodeisoquinoline-1,3(2H)dione,5-amino-2(2-dimethyl-aminoethyl [sic]) (hereinafter "NSC 308847"), was found to show excellent activity against these two specific tumor models. Based on their analysis, compound NSC 308847 was selected for further studies by NCI. In addition to comparing the effectiveness of the claimed compounds [**4] with structurally similar compounds in Paull, applicants' patent specification illustrates the cytotoxicity of the claimed compounds against human tumor cells, ***in vitro***, [5] and concludes that these tests "had a good action." [6]

3 ***In vivo*** means "in the living body, referring to a process occurring therein." **Steadman's Medical Dictionary** 798 (25th ed. 1990). ***In vitro*** means "in an artificial environment, referring to a process or reaction occurring therein, as in a test tube or culture media." ***Id.***

4 The analysis in Paull consisted of grouping the previously-tested compounds into groups based on common structural features and cross-referencing the various groups, in light of the success rates of the group as a whole, to determine specific compounds that may be effective in treating tumors.

5 ***See supra*** note 3.

6 The specification does not state the specific type of human tumor cells used in this test.

The examiner initially rejected applicants' [**5] claims in the '690 application as obvious under 35 U.S.C. § 103 in light of U.S. Patent No. 4,614,820, issued to and referred to hereafter as Zee-Cheng et al. Zee-Cheng et al. discloses a benzodeisoquinoline compound for use as an antitumor agent with symmetrical substitutions on the 5-position and 8-position of the quinoline ring; in both positions the substitution was either an amino or nitro group. [7] Although not identical to the applicants' claimed compounds, the examiner noted the similar substitution pattern (i.e., at the same positions on the isoquinoline ring) and concluded that a mixed substitution of the invention therefore would have been obvious in view of Zee-Cheng et al.

7 The chemical compound in Zee-Cheng et al. is labeled a 3,6-disubstituted-1,8-naphthalimide and uses different numbering for the positions on the isoquinoline ring. The structure of this compound, however, is identical to that claimed by the applicants except for symmetrical substitutions at the 5-position and the 8-position of the isoquinoline ring. Zee-Cheng et al. teaches identical substitutions of amino or nitro groups while applicants claim a nitro group substitution at the 5-position and an amino group substitution at the 8-position.

[**6] In a response dated July 14, 1989, the applicants rebutted the § 103 rejection. Applicants asserted that their mixed disubstituted compounds had unexpectedly better antitumor properties than the symmetrically substituted compounds in Zee-Cheng et al. In support of this assertion applicants attached the declaration of Dr. Gerhard Keilhauer. In his declaration Dr. Keilhauer reported that his tests indicated that applicants' claimed compounds were far more effective as antitumor agents than the compounds disclosed in Zee-Cheng et al. when tested, ***in vitro***, against two specific types of human tumor cells, HEp and HCT-29. [8] Applicants further noted that, although the differences between the compounds in Zee-Cheng et al. and applicants' claimed compounds were slight, there was no suggestion in the art that these improved results (over Zee-Cheng et al.) would have been expected. Although the applicants overcame the § 103 rejection, the examiner nevertheless issued a final rejection, on different grounds, on September 5, 1989.

8 HEp cells are derived from laryngeal cancer and HCT-29 cells from colon cancer.

[**7] On June 4, 1990, applicants filed a continuation application, Serial No. 533,944 (the '944 application), from the above-mentioned '690 application. Claims 10-13, the only claims remaining in the continuation application, were rejected in a final office action dated May 1, 1991. Applicants appealed the examiner's final rejection to the Board.

In his answer to the applicants' appeal brief, the examiner stated that the final rejection was based on 35 U.S.C. § 112 P1. [9] The examiner first noted that the specification failed to describe any specific disease against which the claimed compounds were active. Furthermore, the examiner concluded that the prior art tests performed in Paull and the tests disclosed in the specification were not sufficient to establish a reasonable expectation that the claimed compounds had [*1564] a practical utility (i.e. antitumor activity in humans). [10]

9 The examiner's answer noted that the final rejection also could have been made under 35 U.S.C. § 101 for failure to disclose a practical utility.

10 The examiner subsequently filed two supplemental answers in response to arguments raised by the applicants in supplemental reply briefs.

[**8] In a decision dated March 19, 1993, the Board affirmed the examiner's final rejection. The three-page opinion, which lacked any additional analysis, relied entirely on the examiner's reasoning. Although noting that it also would have been proper for the examiner to reject the claims under 35 U.S.C. § 101, the Board affirmed solely on the basis of the Examiner's § 112 P1 rejection. This appeal followed.

II. DISCUSSION

At issue in this case is an important question of the legal constraints on patent office examination practice and policy. The question is, with regard to pharmaceutical inventions, what must the applicant prove regarding the practical utility or usefulness of the invention for which patent protection is sought. This is not a new issue; it is one which we would have thought had been settled by case law years ago. [11] We note the Commissioner has recently addressed this question in his Examiner Guidelines for Biotech Applications, ***see*** 60 Fed. Reg. 97 (1995); 49 Pat. Trademark & Copyright J. (BNA) No. 1210, at 234 (Jan. 5, 1995).

11 ***See, e.g., Cross v. Iizuka***, 753 F.2d 1040, 224 U.S.P.Q. (BNA) 739 (Fed. Cir. 1985); ***In re Langer***, 503 F.2d 1380, 183 U.S.P.Q. (BNA) 288 (CCPA 1974); ***In re Krimmel***, 48 C.C.P.A. 1116, 292 F.2d 948, 130 U.S.P.Q. (BNA) 215 (CCPA 1961); ***In re Bergel***, 48 C.C.P.A. 1101, 292 F.2d 958, 130 U.S.P.Q. (BNA) 205 (CCPA 1961).

[**9] [HN1]The requirement that an invention have utility is found in 35 U.S.C. § 101: "Whoever invents . . . any new and ***useful*** . . . composition of matter . . . may obtain a patent therefor . . . ." (emphasis added). [HN2]It is also implicit in § 112 P1, which reads:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Obviously, if a claimed invention does not have utility, the specification cannot enable one to use it.

As noted, although the examiner and the Board both mentioned § 101, and the rejection appears to be based on the issue of whether the compounds had a practical utility, a § 101 issue, the rejection according to the Board stands on the requirements of § 112 P1. It is to that provision that we address ourselves. [12] The Board gives two reasons for the rejection; [13] we will consider these in turn.

12 This court's predecessor has determined that absence of utility can be the basis of a rejection under both 35 U.S.C. § 101 and § 112 P1. ***In re Jolles***, 628 F.2d 1322, 1326 n.11, 206 U.S.P.Q. (BNA) 885, 889 n.11 (CCPA 1980); ***In re Fouche***, 58 C.C.P.A. 1086, 439 F.2d 1237, 1243, 169 U.S.P.Q. (BNA) 429, 434 (CCPA 1971)("If such compositions are in fact useless, appellant's specification cannot have taught how to use them."). Since the Board affirmed the examiner's rejection based solely on § 112 P1, however, our review is limited only to whether the application complies with § 112 P1.

[**10]

13 The Board's decision did not expressly make any independent factual determinations or legal conclusions. Rather, the Board stated that it "agreed with the examiner's well reasoned, well stated and fully supported by citation of relevant precedent position in every particular, and any further comment which we might add would be redundant." ***Ex parte Brana et al.***, No. 92-1196 (Bd. Pat. App. & Int. March 19, 1993) at 2-3. Therefore, reference in this opinion to Board findings are actually arguments made by the examiner which have been expressly adopted by the Board.

1.

The first basis for the Board's decision was that the applicants' specification failed to disclose a specific disease against which the claimed compounds are useful, and therefore, absent undue experimentation, one of ordinary skill in the art was precluded from using the invention. ***See Hybritech Inc. v. Monoclonal Antibodies, Inc.***, 802 F.2d 1367, 1384, 231 U.S.P.Q. (BNA) 81, 94 (Fed. Cir. 1986), ***cert. denied***, 480 U.S. 947, 94 L. Ed. 2d 792, 107 S. Ct. 1606 (1987). In support, the Commissioner argues that the disclosed uses in [*1565] the '944 application, [**11] namely the "treatment of diseases" and "antitumor substances," are similar to the nebulous disclosure found insufficient in ***In re Kirk***, 54 C.C.P.A. 1119, 376 F.2d 936, 153 U.S.P.Q. (BNA) 48 (CCPA 1967). This argument is not without merit.

In ***Kirk*** applicants claimed a new class of steroid compounds. One of the alleged utilities disclosed in the specification was that these compounds possessed "high biological activity." 376 F.2d at 938, 153 U.S.P.Q. (BNA) at 50. The specification, however, failed to disclose which biological properties made the compounds useful. Moreover, the court found that known specific uses of similar compounds did not cure this defect since there was no disclosure in the specification that the properties of the claimed compounds were the same as those of the known similar compounds. ***Id.*** at 942, 153 U.S.P.Q. (BNA) at 53. Furthermore, it was not alleged that one of skill in the art would have known of any specific uses, and therefore, the court concluded this alleged use was too obscure to enable one of skill in the art to use the claimed invention. ***See also Kawai v. Metlesics***, 480 F.2d 880, 178 U.S.P.Q. (BNA) 158 (CCPA 1973).

Kirk would potentially be dispositive [**12] of this case were the above-mentioned language the only assertion of utility found in the '944 application. Applicants' specification, however, also states that the claimed compounds have "a better action and a better action spectrum as antitumor substances" than known compounds, specifically those analyzed in Paull. As previously noted, ***see supra*** note 4, Paull grouped various benzodeisoquinoline-1,3-diones, which had previously been tested ***in vivo*** for antitumor activity against two lymphocytic leukemia tumor models (P388 and L1210), into various structural classifications and analyzed the test results of the groups (i.e. what percent of the compounds in the particular group showed success against the tumor models). Since one of the tested compounds, NSC 308847, was found to be highly effective against these two lymphocytic leukemia tumor models, [14] applicants' favorable comparison implicitly asserts that their claimed compounds are highly effective (i.e. useful) against lymphocytic leukemia. An alleged use against this particular type of cancer is much more specific than the vaguely intimated uses rejected by the courts in ***Kirk*** and ***Kawai***. [**13] ***See, e.g., Cross v. Iizuka***, 753 F.2d at 1048, 224 U.S.P.Q. (BNA) at 745 (finding the disclosed practical utility for the claimed compounds -- the inhibition of thromboxane synthetase in human or bovine platelet microsomes -- sufficiently specific to satisfy the threshold requirement in ***Kirk*** and ***Kawai***.)

14 Paull also found NSC 308847 to be effective against two other test models, B16 melanoma and Colon C872.

The Commissioner contends, however, that P388 and L1210 are not diseases since the only way an animal can get sick from P388 is by a direct injection of the cell line. The Commissioner therefore concludes that applicants' reference to Paull in their specification does not provide a specific disease against which the claimed compounds can be used. We disagree.

As applicants point out, the P388 and L1210 cell lines, though technically labeled tumor models, were originally derived from lymphocytic leukemias in mice. Therefore, the P388 and L1210 cell lines do represent actual specific lymphocytic [**14] tumors; these models will produce this particular disease once implanted in mice. If applicants were required to wait until an animal naturally developed this specific tumor before testing the effectiveness of a compound against the tumor ***in vivo***, as would be implied from the Commissioner's argument, there would be no effective way to test compounds ***in vivo*** on a large scale.

We conclude that these tumor models represent a specific disease against which the claimed compounds are alleged to be effective. Accordingly, in light of the explicit reference to Paull, applicants' specification alleges a sufficiently specific use.

2.

The second basis for the Board's rejection was that, even if the specification did allege a specific use, applicants failed to [*1566] prove that the claimed compounds are useful. Citing various references, [15] the Board found, and the Commissioner now argues, that the tests offered by the applicants to prove utility were inadequate to convince one of ordinary skill in the art that the claimed compounds are useful as antitumor agents. [16]

15 ***See*** Pazdur et al., ***Correlation of Murine Antitumor Models in Predicting Clinical Drug Activity in Non-Small Cell Lung Cancer: A Six Year Experience***, 3 **Proceedings Am. Soc. Clin. Oncology** 219 (1984); Martin et al., ***Role of Murine Tumor Models in Cancer Research***, 46 **Cancer Research** 2189 (April 1986).

[**15]

16 As noted, this would appear to be a § 101 issue, rather than § 112.

This court's predecessor has stated:

> [A] specification [HN3]disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented ***must*** be taken as in compliance with the enabling requirement of the first paragraph of § 112 ***unless*** there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

In re Marzocchi, 58 C.C.P.A. 1069, 439 F.2d 220, 223, 169 U.S.P.Q. (BNA) 367, 369 (CCPA 1971). From this it follows that [HN4]the PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure. 439 F.2d at 224, 169 U.S.P.Q. (BNA) at 370. [HN5]Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's [**16] asserted utility. ***See In re Bundy***, 642 F.2d 430, 433, 209 U.S.P.Q. (BNA) 48, 51 (CCPA 1981). [17]

17 ***See also In re Novak***, 49 C.C.P.A. 1283, 306 F.2d 924, 928, 134 U.S.P.Q. (BNA) 335, 337 (CCPA 1962) (stating that it is proper for the examiner to request evidence to substantiate an asserted utility unless one with ordinary skill in the art would accept the allegations as obviously valid and correct); ***In re Chilowsky***, 43 C.C.P.A. 775, 229 F.2d 457, 462, 108 U.S.P.Q. (BNA) 321, 325 (CCPA 1956) ("Where the mode of operation alleged can be readily understood and conforms to the known laws of physics and chemistry . . . no further evidence is required."). ***But see In re Marzocchi***, 439 F.2d at 223, 169 U.S.P.Q. (BNA) at 369-70 ("In the field of chemistry generally there may be times when the well-known unpredictability of chemical reactions will alone be enough to create a reasonable doubt as to the accuracy of a particular broad statement put forward as enabling support for a claim. This will especially be the case where the statement is, on its face, contrary to generally accepted scientific principles.").

[**17] The PTO has not met this initial burden. The references cited by the Board, Pazdur and Martin, [18] do not question the usefulness of any compound as an antitumor agent or provide any other evidence to cause one of skill in the art to question the asserted utility of applicants' compounds. Rather, these references merely discuss the therapeutic predictive value of ***in vivo*** murine tests -- relevant only if applicants must prove the ultimate value in humans of their asserted utility. Likewise, we do not find that the nature of applicants'

invention alone would cause one of skill in the art to reasonably doubt the asserted usefulness.

18 ***See supra*** note 15.

The purpose of treating cancer with chemical compounds does not suggest an inherently unbelievable undertaking or involve implausible scientific principles. ***In re Jolles***, 628 F.2d at 1327, 206 U.S.P.Q. (BNA) at 890. Modern science has previously identified numerous successful chemotherapeutic agents. In addition, the prior art, specifically [**18] Zee Cheng et al., discloses structurally similar compounds to those claimed by the applicants which have been proven ***in vivo*** to be effective as chemotherapeutic agents against various tumor models.

Taking these facts -- the nature of the invention and the PTO's proffered evidence -- into consideration we conclude that one skilled in the art would be without basis to reasonably doubt applicants' asserted utility on its face. The PTO thus has not satisfied its initial burden. Accordingly, applicants should not have been required to substantiate their presumptively correct disclosure to avoid a rejection under the first paragraph of § 112. ***See In re Marzocchi***, 439 F.2d at 224, 169 U.S.P.Q. (BNA) at 370.

We do not rest our decision there, however. Even if one skilled in the art [*1567] would have reasonably questioned the asserted utility, i.e., even if the PTO met its initial burden thereby shifting the burden to the applicants to offer rebuttal evidence, applicants proffered sufficient evidence to convince one of skill in the art of the asserted utility. In particular, applicants provided through Dr. Kluge's declaration [19] test results showing that several compounds within the [**19] scope of the claims exhibited significant antitumor activity against the L1210 standard tumor model ***in vivo***. Such evidence alone should have been sufficient to satisfy applicants' burden.

19 The declaration of Michael Kluge was signed and dated June 19, 1991. This declaration listed test results (i.e. antitumor activity) of the claimed compounds, ***in vivo***, against L1210 tumor cells and concluded that these compounds would likely be clinically useful as anti-cancer agents. Enablement, or utility, is determined as of the application filing date. ***In re Glass***, 492 F.2d 1228, 1232, 181 U.S.P.Q. (BNA) 31, 34 (CCPA 1974). The Kluge declaration, though dated after applicants' filing date, can be used to substantiate any doubts as to the asserted utility since this pertains to the accuracy of a statement already in the specification. ***In re Marzocchi***, 439 F.2d at 224 n.4, 169 U.S.P.Q. (BNA) at 370 n.4. It does not render an insufficient disclosure enabling, but instead goes to prove that the disclosure was in fact enabling when filed (i.e., demonstrated utility).

[**20] The prior art further supports the conclusion that one skilled in the art would be convinced of the applicants' asserted utility. As previously mentioned, prior art -- Zee Cheng et al. and Paull -- disclosed structurally similar compounds which were proven ***in vivo*** against various tumor models to be effective as chemotherapeutic agents. [HN6]Although it is true that minor changes in chemical compounds can radically alter their effects on the human body, ***Kawai***, 480 F.2d at 891, 178 U.S.P.Q. (BNA) at 167, evidence of success in structurally similar compounds is relevant in determining whether one skilled in the art would believe an asserted utility. ***See Rey-Bellet v. Engelhardt***, 493 F.2d 1380, 181 U.S.P.Q. (BNA) 453 (CCPA 1974); ***Kawai***, 480 F.2d 880, 178 U.S.P.Q. (BNA) 158.

The Commissioner counters that such ***in vivo*** tests in animals are only preclinical tests to determine whether a compound is suitable for processing in the second stage of testing, by which he apparently means ***in vivo*** testing in humans, and therefore are not reasonably predictive of the success of the claimed compounds for treating cancer in humans. [20] The Commissioner, as did the Board, confuses [**21] the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption. ***See Scott v. Finney***, 34 F.3d 1058, 1063, 32 U.S.P.Q.2D (BNA) 1115, 1120 (Fed. Cir. 1994) ("Testing for the full safety and effectiveness of a prosthetic device is more properly left to the Food and Drug Administration (FDA). Title 35 does not demand that such human testing occur within the confines of Patent and Trademark Office (PTO) proceedings.").

20 We note that this discussion is relevant to the earlier discussion as well. If we were to conclude that these ***in vivo*** tests are insufficient to establish usefulness for the claimed compounds, that would bear on the issue of whether one skilled in the art would, in light of the structurally similar compounds in Paull and Zee Cheng et al., have cause to doubt applicants' asserted usefulness for the compounds.

Our court's predecessor has determined that [HN7]proof of an alleged pharmaceutical property [**22] for a compound by statistically significant tests with standard experimental animals is sufficient to establish utility. ***In re Krimmel***, 48 C.C.P.A. 1116, 292 F.2d 948, 953, 130 U.S.P.Q. (BNA) 215, 219 (CCPA 1961); ***see also In re Bergel***, 48 C.C.P.A. 1101, 292 F.2d 958, 130 U.S.P.Q. (BNA) 205 (CCPA 1961). In

concluding that similar ***in vivo*** tests were adequate proof of utility the court in ***In re Krimmel*** stated:

We hold as we do because it is our firm conviction that one who has taught the public that a compound exhibits some desirable pharmaceutical property in a standard experimental animal has made a significant and useful contribution to the art, even though it may eventually appear that the compound is without value in the treatment in humans.

Krimmel, 292 F.2d at 953, 130 U.S.P.Q. (BNA) at 219. Moreover, NCI apparently believes these tests are statistically significant because it has explicitly recognized both the P388 and L1210 murine tumor models as standard screening tests for determining whether new [*1568] compounds may be useful as antitumor agents.

In the context of this case the Martin and Pazdur references, on which the Commissioner relies, do not convince us otherwise. Pazdur only questions [**23] the reliability of the screening tests against lung cancer; it says nothing regarding other types of tumors. Although the Martin reference does note that some laboratory oncologists are skeptical about the predictive value of ***in vivo*** murine tumor models for human therapy, Martin recognizes that these tumor models continue to contribute to an increasing human cure rate. In fact, the authors conclude that this perception (i.e. lack of predictive reliability) is not tenable in light of present information.

On the basis of animal studies, and controlled testing in a limited number of humans (referred to as Phase I testing), the Food and Drug Administration may authorize Phase II clinical studies. ***See*** 21 U.S.C. § 355(i)(1); 5 C.F.R. § 312.23(a)(5), (a)(8) (1994). Authorization for a Phase II study means that the drug may be administered to a larger number of humans, but still under strictly supervised conditions. The purpose of the Phase II study is to determine primarily the safety of the drug when administered to a larger human population, as well as its potential efficacy under different dosage regimes. ***See*** 21 C.F.R. § 312.21(b).

[HN8]FDA approval, however, [**24] is not a prerequisite for finding a compound useful within the meaning of the patent laws. ***Scott***, 34 F.3d 1058, 1063, 32 U.S.P.Q.2D (BNA) 1115, 1120. [HN9]Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.

In view of all the foregoing, we conclude that applicants' disclosure complies with the requirements of 35 U.S.C. § 112 P1.

3.

The Commissioner takes this opportunity to raise the question of this court's standard of review when deciding cases on appeal from the PTO. Traditionally we have [HN10]recited our standard of review to be, with regard to questions of law, that review is without deference to the views of the Agency, ***In re Donaldson***, [**25] 16 F.3d 1189, 1192, 29 U.S.P.Q.2D (BNA) 1845, 1848 (Fed. Cir. 1994) (in banc), ***In re Caveney***, 761 F.2d 671, 674, 226 U.S.P.Q. (BNA) 1, 3 (Fed. Cir. 1985), and with regard to questions of fact, we defer to the Agency unless its findings are "clearly erroneous." ***See, e.g., In re Baxter Travenol Labs***, 952 F.2d 388, 21 U.S.P.Q.2D (BNA) 1281 (Fed. Cir. 1991); ***In re Woodruff***, 919 F.2d 1575, 16 U.S.P.Q.2D (BNA) 1934 (Fed. Cir. 1990); ***In re De Blauwe***, 736 F.2d 699, 222 U.S.P.Q. (BNA) 191 (Fed. Cir. 1984).

[HN11]With regard to judgment calls, those questions that fall "somewhere near the middle of the fact-law spectrum," this court has recognized "the falseness of the fact-law dichotomy, since the determination at issue, involving as it does the application of a general legal standard to particular facts, is probably most realistically described as neither of fact nor law, but mixed." ***Campbell v. Merit Systems Protection Board***, 27 F.3d 1560, 1565 (Fed. Cir. 1994). When these questions of judgment are before us, whether we defer, and the extent to which we defer, turns on the nature of the case and the nature of the judgment. ***Id.*** ("Characterization therefore must follow from an *a* [**26] *priori* decision as to whether deferring . . . is sound judicial policy. We would be less than candid to suggest otherwise.").

The Commissioner contends that the appropriate standard of review for this court regarding questions of law, of fact, and mixed questions of law and fact, coming to us from the PTO is found in the Administrative Procedure Act (APA) at 5 U.S.C. § 706. The standard set out there is that "the reviewing court shall . . . hold unlawful and set aside agency action, findings, and conclusions found to be -- (A) arbitrary, capricious, an [*1569] abuse of discretion, or otherwise not in accordance with law; . . . (E) unsupported by substantial evidence . . . ." The Commissioner is of the view that the stated standard we now use, which is the traditional

standard of review for matters coming from a trial court, is not appropriate for decisions coming from an agency with presumed expertise in the subject area, and is not in accord with law. [21]

> 21 Congress enacted the Administrative Procedure Act (APA) on June 11, 1946. ***See*** 1 **Kenneth Culp Davis, Administrative Law Treatise**, § 1:7 (2d ed. 1978). The APA sets forth a framework for administrative agency procedure and provides judicial review for persons adversely affected by final agency actions. Chapter 7, codified at 5 U.S.C. § 701-706, contains the APA judicial review provisions, including the standard of review provision quoted above.

[**27] Applicants argue that by custom and tradition, recognized by the law of this court, the standard of review we have applied, even though inconsistent with the standard set forth in the APA, nevertheless is a permissible standard. In our consideration of this issue, there is a reality check: would it matter to the outcome in a given case which formulation of the standard a court articulates in arriving at its decision? The answer no doubt must be that, even though in some cases it might not matter, in others it would, otherwise the lengthy debates about the meaning of these formulations and the circumstances in which they apply would be unnecessary.

A preliminary question, then, is whether this is one of those cases in which a difference in the standard of review would make a difference in the outcome. The ultimate issue is whether the Board correctly applied the § 112 P1 enablement mandate and its implicit requirement of practical utility, or perhaps more accurately the underlying requirement of § 101, to the facts of this case. As we have explained, the issue breaks down into two subsidiary issues: (1) whether a person of ordinary skill in the art would conclude that the applicants [**28] had sufficiently described particular diseases addressed by the invention, and (2) whether the Patent Act supports a requirement that makes human testing a prerequisite to patentability under the circumstances of this case.

The first subsidiary issue, whether the application adequately described particular diseases, calls for a judgment about what the various representations and discussions contained in the patent application's specification would say to a person of ordinary skill in the art. We have considered that question carefully, and, for the reasons we explained above in some detail, we conclude that the Board's judgment on this question was erroneous. Our conclusion rests on our understanding of what a person skilled in the art would gather from the various art cited, and from the statements in the application itself. We consider the Board's error to be sufficiently clear that it is reversible whether viewed as clear error or as resulting in an arbitrary and capricious decision.

The second subsidiary issue, whether human testing is a prerequisite to patentability, is a pure question of law: what does the practical utility requirement mean in a case of this kind. Under either [**29] our traditional standard or under the APA standard no deference is owed the Agency on a question of law, and none was accorded.

If the question concerning the standard of review, raised by the Commissioner, is to be addressed meaningfully, it must arise in a case in which the decision will turn on that question, and, recognizing this, the parties fully brief the issue. This is not that case. We conclude that it is not necessary to the disposition of this case to address the question raised by the Commissioner; accordingly, we decline the invitation to do so.

III. CONCLUSION

The Board erred in affirming the examiner's rejection under 35 U.S.C. § 112 P1. The decision is reversed.

REVERSED

PETER E. CROSS, ET AL., Appellants v. KINJI IIZUKA, ET AL., Appellees

No. 84-1111

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

753 F.2d 1040; 1985 U.S. App. LEXIS 14694; 224 U.S.P.Q. (BNA) 739

January 28, 1985

PRIOR HISTORY: [**1] Appealed from United States Patent & Trademark Office.

CASE SUMMARY:

PROCEDURAL POSTURE: Appellant sought review of the decision of the United States Patent and Trademark Office Board of Patent Interferences awarding priority on a count to appellee in determination of applications filed by both parties under 35 U.S.C.S. § 119.

OVERVIEW: Appellant and appellee submitted patent applications to the Board for priority of a pharmacological compound, each moving to be accorded a foreign priority application under 35 U.S.C.S. § 119 and asserting the other's application did not comply with the disclosure requirements of 35 U.S.C.S. § 112. Because appellee filed the priority application first, appellee was declared the senior party and the Board held appellee's application contained an adequate how-to-use disclosure for the practical utility stated therein. Appellant sought review. The court held that where the Board was charged with the factual determination of utility and found the specifications of appellee's application disclosed the compound's utility and where credible evidence to support that factual determination existed, the determination would be upheld. As appellant bore the burden of proof to show that the Board erred in finding appellee's priority application adequately disclosed a practical utility and failed to do so, the Board's determination that appellee's application had a practical utility was upheld.

OUTCOME: The court affirmed the judgment.

LexisNexis(R) Headnotes

Patent Law > Utility Requirement > Proof of Utility
[HN1]An invention cannot be considered useful, in the sense that a patent can be granted on it, unless substantial or practical utility for the invention has been discovered and disclosed where such utility would not be obvious.

Patent Law > Utility Requirement > Proof of Utility
[HN2]Where a constructive reduction to practice is involved, as contrasted to an actual reduction to practice, a practical utility for the invention is determined by reference to, and a factual analysis of, the disclosures of the application.

Patent Law > Utility Requirement > Proof of Utility
[HN3]Evidence of any utility is sufficient when the count does not recite any particular utility.

Patent Law > Utility Requirement > Proof of Utility
[HN4]A consideration in the determination of whether a patent should be granted is the benefit derived by the public from an invention with substantial utility. Unless and until a process is refined and developed to this point -- where specific benefit exists in currently available form -- there is insufficient justification for permitting an applicant to engross what may prove to be a broad field.

Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
Patent Law > Utility Requirement > Chemical Compounds
Patent Law > Utility Requirement > Proof of Utility
[HN5]Knowledge of the pharmacological activity of any compound is obviously beneficial to the public and adequate proof of any such utility constitutes a showing of practical utility.

Patent Law > Utility Requirement > Proof of Utility
[HN6]Where a count contains no limitation related to utility, evidence establishing a substantial utility for any purpose is sufficient to show a reduction to practice.

Patent Law > Utility Requirement > Chemical Compounds
[HN7]Every utility question arising in an interference, in the final analysis, must be decided on the basis of its own unique factual circumstances. Relevant evidence must be judged as a whole for its persuasiveness in determining whether the suggested use for the compound of the count is a practical utility.

Patent Law > Utility Requirement > Chemical Compounds
[HN8]A particular pharmacological activity identified with prior art compounds may have probative value as to the fact that the compound of the count possesses this particular pharmacological activity where there is a structural similarity between the prior art compounds and the compound of the count.

Patent Law > Utility Requirement > Chemical Compounds
Patent Law > Utility Requirement > Proof of Utility
[HN9]Adequate proof of any pharmacological activity constitutes a showing of practical utility.

COUNSEL: Rudolf E. Hutz, Connolly, Bove, Lodge & Hutz, of Wilmington, Delaware, argued for Appellants. With him on the brief was Thomas M. Meshbesher.

Peter D. Olexy, Sughrue, Mion, Zinn, MacPeak, & Seas, of Washington, District of Columbia, argued for Appellees. With him on the brief was Thomas J. MacPeak.

JUDGES: Kashiwa, Bennett, and Bissell, Circuit Judges.

OPINION BY: KASHIWA

OPINION

[*1041] KASHIWA, Circuit Judge.

This appeal is from the decision of the United States Patent and Trademark Office (PTO) Board of Patent Interferences (Board) awarding priority on the single phantom count to Iizuka, *et al.* (Iizuka), the senior party. We affirm.

Background

Interference No. 100,650 was declared on 20 April 1981 between application serial No. 68,365, for "Imidazole Derivatives," filed by Iizuka on 21 August 1979 and application serial No. 95,755, for "N-(Phenoxyalkyl) Imidazoles as Selective Inhibitors of the Thromboxane Synthetase Enzyme and Pharmaceutical Compositions [*1042] Thereof," filed by Cross, *et al.* (Cross) on 19 November 1979. The single phantom count of the interference is directed to imidazole [**2] derivative compounds and reads as follows:

> A compound selected from the group consisting of an imidazole derivative of the formula
>
> [SEE ILLUSTRATION IN ORIGINAL]
>
> wherein R is a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, each of A[1] or A[2], which may be the same or different, are alkylene having 1 to 8 carbon atoms, m is 0 or 1, X is oxygen or sulfur, and each of R[1] or R[2], which may be the same or different, is a hydrogen atom or an alkyl group having 1 to 6 carbon atoms; R[3] is H, C[1]-C[4] alkyl, C[1]-C[4] alkoxy or halogen; and the pharmaceutically acceptable salts thereof. [1]

1 We note a discrepancy, shown underlined in the above count, between the phantom count as set forth by the primary examiner and that reported by the Board in its decision. The phantom count set forth herein is the one propounded by the primary examiner. However, as will become apparent from the ensuing discussion, the substance of the phantom count is

not crucial to resolution of the issues presented by this case.

[**3] The applications of Cross and Iizuka both disclose inventions directed to imidazole derivative compounds which inhibit the synthesis of thromboxane synthetase, an enzyme which leads to the formation of thromboxane A[2] (TXA[2]), [2] a highly unstable, biologically active compound which is converted to stable thromboxane B[2] by the addition of water. Thromboxane A[2], as of the time period during which the applications were filed, was postulated to be a causal factor in platelet aggregation. [3] Platelet aggregation is associated with several deleterious conditions in mammalia, including humans, such as platelet thrombosis, pulmonary vasoconstriction or vasospasm, inflammation, hypertension, and collagen-induced thrombosis.

2 The formation of TXA[2] in an arachidonic acid challenge is a sequential process initiated by the conversion of arachidonic acid to prostaglandin PGG[2] by the action of cyclooxygenase, which adds oxygen to the acid. Peroxidase converts the prostaglandin PGG[2] to prostaglandin PGH[2], which in turn is converted by thromboxane synthetase to TXA[2].

[**4]

3 Iizuka's position is that, as of the "critical date" of his application, TXA[2] was widely accepted in the art as causing platelet aggregation. Cross' position is that, as of the "critical date," platelet aggregation was believed to be nonspecific, i.e., platelet aggregation *may* occur in the presence of thromboxane synthetase, but thromboxane synthetase is not necessary for platelet aggregation. We note in retrospect that THE MERCK INDEX 1345-46 (10th ed. 1983) describes TXA[2] as inducing irreversible platelet aggregation. More to the point, however, this court has noted that it is axiomatic that an inventor need not comprehend the scientific principles on which the practical effectiveness of his invention rests, nor is the inventor's theory or belief as to how his invention works a necessary element in the specification to satisfy the enablement requirement of 35 U.S.C. § 112. *Fromson v. Advance Offset Plate, Inc.*, 720 F.2d 1565, 1570, 219 U.S.P.Q. (BNA) 1137, 1140 (Fed. Cir. 1983).

Pursuant to 37 C.F.R. § 1.231(a)(4) each party moved to be accorded the benefit of the [**5] foreign priority application under 35 U.S.C. § 119, Cross claiming priority based upon a British application filed 13 December 1978, and Iizuka claiming priority based upon a Japanese application filed 21 August 1978. Each party opposed the motion of the other party, each party contending that the other party's foreign priority application did not comply with the disclosure requirements of 35 U.S.C. § 112.

The primary examiner granted each party's motion, noting that the utility alleged in each application was of a pharmacological nature, i.e., the inhibition of thromboxane synthetase, and that inasmuch as the single phantom count of the interference was directed to a compound, it was not necessary that utility be established by tests and dosages with respect to human beings. The examiner found that one of ordinary skill in the art would know how to use the imidazole derivatives, i.e., be able to determine specific dosages, for biological purposes. Based upon the filing dates of [*1043] the foreign priority applications, [4] Iizuka was declared the senior party and a show cause order was issued against Cross.

4 Each party relies on the filing date of its foreign priority application to establish a constructive reduction to practice, the earliest date of invention to which each party is entitled under the patent laws of the United States. *Kawai v. Metlesics*, 480 F.2d 880, 885-86, 178 U.S.P.Q. (BNA) 158, 162 (CCPA 1973).

[**6] Cross requested a final hearing on the issue of the sufficiency of the Japanese priority application of Iizuka, and moved for a testimony period to present evidence on this issue. A testimony period was granted over the opposition of Iizuka, and Cross took the testimony of his expert witness, Dr. Smith, and Iizuka took the testimony of his expert witness, Dr. Ramwell and also proffered several exhibits pursuant to 37 C.F.R. § 1.282. All testimony and exhibits related to the sufficiency of Iizuka's Japanese priority application, i.e., whether it complied with the disclosure requirements of 35 U.S.C. § 112.

Decision of the Board

The Board noted that the sole issue before it was whether Iizuka was entitled to the benefit of his Japanese priority application. [5] Relying on *In re Bundy*, 642 F.2d 430, 209 U.S.P.Q. (BNA) 48 (CCPA 1981), and *Nelson v. Bowler*, 626 F.2d 853, 206 U.S.P.Q. (BNA) 881 (CCPA 1980), the Board held that tests evidencing pharmacological activity may manifest a practical utility even though they may not establish a specific therapeutic use. The Board found that the Japanese priority application disclosed pharmacological activity in the similar [**7] activity of the imidazole derivatives of the count to imidazole and 1-methylimidazole, which possess an inhibitory action for thromboxane synthetase, and that practical utility was disclosed in the strong

inhibitory action for thromboxane synthetase from human or bovine platelet microsomes, i.e., an *in vitro* utility. [6]

> 5 More specifically, the issue before the Board was whether the Japanese priority application complied with the how-to-use requirement of 35 U.S.C. § 112. Section 112 of Title 35 provides, in pertinent part, that:
>
> > The specification shall contain a written description of the invention, of the manner and process of making and *using* it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and *use* the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention. (Emphasis added.)
>
> Should Iizuka's Japanese priority application be found nonenabling with respect to the how-to-use requirement of § 112, or otherwise found deficient under the patent laws of the United States, priority would be awarded to Cross based upon his unchallenged entitlement to the benefit of his British application.

[**8]

> 6 Generally, *in vitro* refers to an environment outside of a living organism, usually an artificial environment such as a test tube or culture. In contradistinction, *in vivo* generally refers to an environment within a living organism, such as a plant or animal, or it may refer to a particular portion of an organ external to the living organism, e.g., rat aortic loop.

The Board further found that the Japanese priority application disclosed "how-to-use" knowledge directed to the practical utility in a microsome system, and that microsome assays were admittedly known in the art. A skilled worker could determine the relative strength of the imidazole compounds of the count vis-a-vis the known parent imidazole and 1-methylimidazole compounds for use in the microsome assay milieu. Knowledge of the pharmacological activities of compounds is beneficial to the medical profession, and requiring Iizuka to have disclosed *in vivo* dosages in the Japanese priority application would delay and frustrate researchers by failing to provide an incentive for early public disclosure of such compounds, [**9] thereby failing to further the public interest.

Accordingly, the Board held that the Japanese priority application contained an adequate how-to-use disclosure for the practical utility stated therein.

Issues

Whether the Board erred in finding that the utility disclosed in the Japanese priority application is sufficient to meet the practical utility requirement of 35 U.S.C. § 101.

[*1044] Whether the Board erred in finding that the Japanese priority application contained sufficient disclosure to satisfy the enablement, i.e., how-to-use, requirement of 35 U.S.C. § 112. [7]

> 7 Utility is a fact question. *Raytheon Co. v. Roper Corp.*, 724 F.2d 951, 956, 220 U.S.P.Q. (BNA) 592, 596 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835, 105 S. Ct. 127, 83 L. Ed. 2d 69, 225 U.S.P.Q. (BNA) 232 (1984). Enablement under § 112, paragraph 1, i.e., the how-to-use requirement, is a question of law. *Id.* at 960 n.6, 220 U.S.P.Q. (BNA) at 599 n.6.

OPINION

Proper resolution of the issues before this [**10] court necessitates that we address, *seriatim*, the following questions: (1) What utility is disclosed by the Japanese priority application? (2) Does this stated utility comply with the "practical utility" requirement of 35 U.S.C. § 101, as delimited by prior decisions of the judiciary? [8] (3) Does the Japanese priority application contain sufficient disclosure to meet the how-to-use requirement of § 112 with respect to the stated utility?

> 8 While questions one and two are closely connected, a thorough analysis of the utility issue requires first, a determination as to what utility is disclosed, i.e., the stated utility, for the invention claimed in the application. Only after the stated utility has been determined, can a proper analysis be undertaken to determine if the stated utility complies with the "practical utility" requirement of § 101. As noted above, these questions regarding utility are factual in nature, *see supra* note 7, and are to be determined in the first instance by the PTO, the agency with the expertise in this regard.

[**11] It is axiomatic that [HN1]an invention cannot be considered "useful", in the sense that a patent can be granted on it, unless substantial or practical utility for the invention has been discovered and disclosed where such utility would not be obvious. *Brenner v.*

Manson, 383 U.S. 519, 16 L. Ed. 2d 69, 86 S. Ct. 1033, 148 U.S.P.Q. (BNA) 689 (1966). [HN2]Where a constructive reduction to practice is involved, as contrasted to an actual reduction to practice, a practical utility for the invention is determined by reference to, and a factual analysis of, the disclosures of the application. *Kawai v. Metlesics*, 480 F.2d 880, 178 U.S.P.Q. (BNA) 158 (CCPA 1973).

1. Japanese Priority Application

The Board factually analyzed the Japanese priority application and found that the only effective disclosure relating to a stated utility for the imidazole derivative compounds of the phantom count was the following:

> [The compounds disclosed] are useful for treatment of inflammation, thrombus, hypertension, cerebral apoplexy, asthma, etc.
>
> Up to this time, it is a known fact that imidazole and 1-methylimidazole possess an inhibitory action for thromboxane synthetase and inhibit a biosynthesis [**12] of thromboxane A[2]. (*Prostaglandins*, Vol. 13, pages 611-, *1977*). However, since their inhibitory effect is not satisfactory one, these compounds have not been put to practical use yet as therapeutical medicines for diseases caused by thromboxane A[2], such as inflammation, hypertension, thrombus, cerebral apoplexy, asthma, etc.
>
> To develop some compounds possessing a strong inhibitory action for biosynthesis of thromboxane A[2], the present inventors devoted themselves to study for various imidazole derivatives, and as a result, found that the compounds [of this invention] possess a strong inhibitory action for thromboxane synthetase from human or bovine platelet microsomes and are extremely useful as therapeutically active agents for diseases caused by thromboxane A[2], for example, inflammation, hypertension, thrombus, cerebral apoplexy, asthma, etc., and thus we proposed this invention based upon those findings.
>
> * * *
>
> The imidazole derivatives . . . of this invention are novel compounds which are not described in literature, and which possess a strong inhibitory action for thromboxane synthetase from human or bovine platelet microsomes, and which [*1045] [**13] exhibit a strong inhibitory action for biosynthesis of thromboxane A[2] in mammalia including human. In general, a satisfactory inhibitory effect is found at a level of molar concentrations of 2.5 x 10<-8>, for example, 2-[p-(1-imidazolylmethyl) phenoxy]-acetic acid hydrochloride produce the about 50% inhibitory effect at the molar concentrations of 2.5 x 10<-8>. Accordingly, the imidazole derivatives of this invention are extremely useful as therapeutical medicines for diseases caused by thromboxane A[2], such as inflammation, hypertension, thrombus, cerebral apoplexy, asthma, etc.

The Board found that these pertinent sections of the Japanese priority application disclosed some activity or utility, namely that the imidazole derivative compounds of the count possess a strong inhibitory action for thromboxane synthetase in human or bovine platelet microsomes. Cross' position is that the stated purpose or *sole* contemplated utility of the invention of Iizuka is to provide a novel class of compounds which provide "practical use" as "therapeutical medicines for diseases caused by thromboxane A[2]," and therefore the Board erred in its finding as to the stated utility [**14] of the Japanese priority application.

While recognizing that *Kawai* constrains an applicant to entitlement to the benefit of only what is disclosed in the foreign priority application and no more, we also recognize that foreign priority applications, as subsequently filed in the PTO, typically have a style and format dissimilar to the arrangement of application elements suggested by 37 C.F.R. § 1.77. In part this arises because of differences in filing requirements in foreign patent offices, and in part because of the awkwardness resulting from direct literal translations from a foreign language to English. Thus, while the factual determination of the stated utility in an application prepared in the United States may be relatively straightforward, [9] the factual analysis of a foreign priority application to determine the utility disclosed therein may be more laborious and open to varying interpretations.

> 9 In applications prepared in the United States by experienced patent drafters, the drafter of the application typically sets forth objectives for the invention in the "Summary of the Invention"

section of the application. These objectives will normally be consonant with the utility disclosed for the invention. As this court has noted, "when a properly claimed invention meets at least one stated objective, utility under § 101 is clearly shown." *Raytheon Co. v. Roper Corp.*, 724 F.2d 951, 958, 220 U.S.P.Q. (BNA) 592, 598 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835, 105 S. Ct. 127, 83 L. Ed. 2d 69 (1984).

[**15] The weakness of Cross' position is that a fair reading of the pertinent sections of the Japanese priority application, as set forth above, discloses utility for the imidazole derivative compounds of the phantom count both as an inhibiting agent for thromboxane synthetase in human or bovine platelet microsomes, as found by the Board, and as therapeutically active agents preventing the biosynthesis of thromboxane A[2], thereby functioning as a medicine preventing deleterious conditions caused by thromboxane A[2], as contended by Cross.

[HN3]Evidence of any utility is sufficient when the count does not recite any particular utility. *Nelson v. Bowler*, 626 F.2d 853, 856, 206 U.S.P.Q. (BNA) 881, 883 (CCPA 1980). *See also Rey-Bellet v. Engelhardt*, 493 F.2d 1380, 181 U.S.P.Q. (BNA) 453 (CCPA 1974); *Knapp v. Anderson*, 477 F.2d 588, 177 U.S.P.Q. (BNA) 688 (CCPA 1973); *Blicke v. Treves*, 44 C.C.P.A. 753, 241 F.2d 718, 112 U.S.P.Q. (BNA) 472 (CCPA 1957). Here the Board, which is charged with the factual determination of utility, [10] has found that the specification of the Japanese priority application disclosed *a* utility for the imidazole derivative compounds of the phantom [**16] count in the inhibition of thromboxane [*1046] synthetase in human or bovine platelet microsomes. Inasmuch as the Board is charged with making this factual determination when the issue is raised, inasmuch as they have so done in the instant case, and inasmuch as there is credible evidence to support this factual determination, we are not prepared to say that the Board erred in its finding as to the stated utility disclosed in the Japanese priority application.

10 Under the facts of the instant case, utility and enablement questions are ancillary to priority. In the interference proceeding, Cross raised the issue as to whether the Japanese priority application contained sufficient disclosure to satisfy § 112. As noted above, *see supra* note 5, if Cross prevails on this issue the Japanese priority application would be removed as the basis for awarding priority to Iizuka. *See generally* 37 C.F.R. §§ 1.225,.231,.258.

2. Practical Utility

As noted in the preceding part of this opinion, Cross [**17] has contended that the Board erred in its finding as to the utility disclosed in the Japanese priority application. This argument may be viewed in a different perspective, we believe, which is that the stated utility in the Japanese priority application, as found by the Board -- the inhibition of thromboxane synthetase in human or bovine platelet microsomes [11] -- is not sufficiently correlated to a pharmacological activity [12] to be a practical utility. In other words, Cross may be arguing that the minimum acceptable level of utility disclosed in an application claiming a compound having pharmacological activity must be directed to an *in vivo* utility in order to comply with the practical utility requirement of § 101.

11 A platelet microsome is an *in vitro* milieu consisting of blood platelets, the small, colorless corpuscles in the blood of all mammals, and other finely granular elements of protoplasm, such as ribosomes, fragmented endoplasmic reticula and mitochondrial christae.

12 Generally, pharmacological activity refers to the properties and reactions of drugs, especially with relation to their therapeutic value.

[**18] The starting point for a practical utility analysis is *Brenner v. Manson*, 383 U.S. 519, 16 L. Ed. 2d 69, 86 S. Ct. 1033, 148 U.S.P.Q. (BNA) 689 (1966). The Court in *Brenner* noted that "a simple, everyday word ["useful," as found in 35 U.S.C. § 101] can be pregnant with ambiguity when applied to the facts of life." *Id.* at 529, 148 U.S.P.Q. (BNA) at 693. [HN4]While noting that "one of the purposes of the patent system is to encourage dissemination of information concerning discoveries and inventions," *id.* at 533, 148 U.S.P.Q. (BNA) at 695, the Court found that a more compelling consideration in the determination of whether a patent should be granted "is the benefit derived by the public from an invention with substantial utility. Unless and until a process is refined and developed to this point -- where specific benefit exists in currently available form -- there is insufficient justification for permitting an applicant to engross what may prove to be a broad field." *Id.* at 534-35, 148 U.S.P.Q. (BNA) at 695. While we recognize that this case concerned a compound derived from a chemical process, we believe *Brenner* provides broad guidelines which are helpful in [**19] ascertaining what constitutes practical utility for compounds having a pharmacological effect.

[HN5]In *Nelson v. Bowler*, 626 F.2d 853, 206 U.S.P.Q. (BNA) 881 (1980), our predecessor court, the Court of Customs and Patent Appeals, stated that "knowledge of the pharmacological activity of any compound is obviously beneficial to the public" and

concluded that "adequate proof of any such utility constitutes a showing of practical utility." *Id.* at 856, 206 U.S.P.Q. (BNA) at 883. [13] The tests [14] found by the court to be adequate proof of pharmacological activity or practical utility were a rat blood pressure (BP) test and a gerbil colon smooth muscle stimulation (GC-SMS) test. The BP test was an *in vivo* test, which was deemed by the court to be direct evidence as to the claimed [*1047] activity, while the GC-SMS test was an *in vitro* test. [15]

13 For purposes of the present opinion, we consider the phrase "substantial utility," as enunciated in *Brenner*, to be synonymous with the phrase "practical utility" as used in subsequent opinions of the CCPA.

14 We recognize that *Nelson* dealt with tests which were found adequate to establish an actual reduction to practice, as opposed to a constructive reduction to practice. We agree with the Board that principles applicable to a determination of an actual reduction to practice are generally germane to a constructive reduction to practice.

[**20]

15 Both parties admitted that the GC-SMS test adequately simulated *in vivo* smooth muscle stimulation.

The CCPA in *Rey-Bellet v. Engelhardt*, 493 F.2d 1380, 1383, 181 U.S.P.Q. (BNA) 453, 454 (1974), stated that [HN6]where a count contains no limitation related to utility, evidence establishing a substantial utility for any purpose is sufficient to show a reduction to practice. The court held that three *in vivo* tests [16] conducted in the United States prior to the filing of Englehardt's U.S. application failed to establish an actual reduction to practice. The court proceeded, however, to find sufficient evidence in the record to establish that Englehardt had conceived a utility for his compound prior to the filing date of his U.S. application. The evidence the court found to be sufficient was testimony by the inventor that he believed his compound would exhibit a particular pharmacological activity because of its structural similarity to another compound which was known to possess the particular pharmacological activity. The court found that the testimonial evidence of Englehardt [**21] was corroborated by two exhibits entered into evidence. The evidence adduced by Englehardt was found by the court to be sufficient proof that Englehardt had conceived that his compound had utility for the particular pharmacological activity prior to his U.S. filing date. The court further noted that this was a completed conception of utility because it appeared that nothing beyond the exercise of routine skill would have been required to demonstrate that Englehardt's compound possessed the particular pharmacological utility. While noting that the actual testing done was not sufficient to establish an actual reduction to practice, the court found that the extensive testing done *in vivo* on animals was routine in nature and was not, therefore, to be construed as an indicator that extensive research, i.e., inventive skill and/or undue experimentation, was required to resolve perplexing intricate difficulties related to the utilization of the compound for the particular pharmacological activity.

16 The three tests, all *in vivo* type tests carried out on laboratory animals, were: (1) the Mental Health General Screening Test which indicated the physical response, or absence of a response, of test animals to a drug, indicating the presence, or absence, of a desired pharmacological activity; (2) the Tetrabenazine Antagonism Test which screened drugs for antidepressant activity; and (3) the Sidman Avoidance Test which screened drugs for tranquilizing activity.

[**22] The CCPA in *Kawai v. Metlesics*, 480 F.2d 880, 178 U.S.P.Q. (BNA) 158 (1973), concurred with the finding of the Board that the applicants had failed to prove that their foreign priority application was adequate under the patent laws of the United States. The only disclosure in the foreign priority application relating to the compound of the count was that it exhibited "pharmacological effects on the central nervous system," which the applicants conceded was an inadequate disclosure. The applicants, however, relied upon a patent made of record as indicative of the general knowledge of one skilled in the art, which the applicants contended described a compound closely related to their claimed compound, to show utility or pharmacological activity for the compound of the count as an anticonvulsant. The court agreed with the board that there were sufficient structural dissimilarities between the compounds of the patent and those of the count to preclude reliance on the patent to supplement the disclosure deficiencies of the foreign priority application.

In *Knapp v. Anderson*, 477 F.2d 588, 177 U.S.P.Q. (BNA) 688 (CCPA 1973), the court, citing to *Blicke v. Treves*, 44 C.C.P.A. [**23] 753, 241 F.2d 718, 112 U.S.P.Q. (BNA) 472 (CCPA 1957), stated that "it is well settled that if the counts do not specify any particular use, evidence proving *substantial utility for any purpose* is sufficient to establish an actual reduction to practice." *Id.* at 590, 177 U.S.P.Q. (BNA) at 690 (emphasis added). Noting that the only utility contemplated for the compounds of the count was as ashless dispersants in lubricant compositions used in internal combustion engines, the court found no error in the Board's holding that there was no actual reduction to practice because [*1048] only a potential utility had been established, this holding based upon the Board's finding of a lack of

correlation between bench tests and actual service conditions in a combustion engine.

The CCPA has held that nebulous expressions, such as "biological activity" or "biological properties," disclosed in a specification convey little explicit indication regarding the utility of a compound. *In re Kirk*, 54 C.C.P.A. 1119, 376 F.2d 936, 941, 153 U.S.P.Q. (BNA) 48, 52 (CCPA 1967). But, while agreeing with the Board that the specification failed to disclose a specific allegation of utility for [**24] any compound within the scope of the claims, and that reference in the specification to biological properties of the claimed compound was so general and vague as to be meaningless, the court implied that a disclosure in the specification that the requisite properties of the claimed compounds are similar to those of a natural or synthetic hormone of known activity may, in appropriate circumstances, supplement an application to rectify an inadequate disclosure relating to the practical utility for the compound. *Id.* at 942, 153 U.S.P.Q. (BNA) at 53.

[HN7]Every utility question arising in an interference, in the final analysis, must be decided on the basis of its own unique factual circumstances. Relevant evidence must be judged as a whole for its persuasiveness in determining whether the suggested use for the compound of the count is a practical utility. *Cf. Nelson*, 626 F.2d at 858, 206 U.S.P.Q. (BNA) at 885.

The Board has found that the Japanese priority application of Iizuka disclosed a practical utility for the compounds of the phantom count in the inhibition of thromboxane synthetase in human or bovine platelet microsomes, i.e., an *in vitro* utility. Clearly, this stated [**25] utility as found by the Board has been delimited with sufficient specificity to satisfy the threshold requirements of *Kawai* and *Kirk*. The stated utility of the Japanese priority application is directed to a specific pharmacological activity possessed by the imidazole derivatives of the phantom count -- the inhibition of thromboxane synthetase *in vitro*. Thus, this court on review is not presented with a general allegation of "biological activity" or "biological properties" as was the CCPA in *Kirk*, nor is reliance on prior art required to ascertain what specific pharmacological activity the compound of the count possesses, the factual situation confronting the court in *Kawai*.

The Japanese priority application, moreover, disclosed that it was generally known in the art, as of the critical date, that the parent imidazole and 1-methylimidazole compounds possessed an inhibitory action for thromboxane synthetase. Reliance on this disclosure in the specification of the pharmacological property of the parent imidazole and 1-methylimidazole compounds, as going towards proof of the pharmacological activity of the imidazole derivatives of the phantom count, is particularly [**26] relevant in the instant case, we believe, because Iizuka is not relying on this inference to supplement an inadequate disclosure in the Japanese priority application regarding the pharmacological activity of the compound of the phantom count, but rather is relying on this inference as cumulative probative evidence showing an adequately disclosed practical utility in the Japanese priority application.

This court, in *Rey-Bellet* and *Kawai*, has implied that [HN8]a particular pharmacological activity identified with prior art compounds may have probative value as to the fact that the compound of the count possesses this particular pharmacological activity where there is a structural similarity between the prior art compounds and the compound of the count. *Rey-Bellet*, 493 F.2d at 1385-87, 181 U.S.P.Q. (BNA) at 456-58; *Kawai*, 480 F.2d at 890-91, 178 U.S.P.Q. (BNA) at 166-67. Cross has failed to proffer sufficient evidence or present any persuasive arguments going to the question of significant structural dissimilarities between the parent imidazole and 1-methylimidazole compounds and the imidazole derivatives of the phantom count. [17]

17 Contrary to Cross' contention in the Reply Brief, the evidence of record relied upon by Cross to show significant structural dissimilarity appears to us to be directed to the fact that there is a wide disparity in potency for thromboxane synthetase inhibition between the parent imidazole compound and prior art imidazole derivatives. Cross has not directed our attention to any specific evidence of record which establishes, or tends to establish, significant structural dissimilarities between the basic imidazole compound and the imidazole derivatives of the phantom count. Variation in potency, moreover, is a matter of degree of activity, *see Bundy*, 642 F.2d at 433, 209 U.S.P.Q. (BNA) at 51, but is still indicative of activity. There is no requirement that the compounds have the same degree of activity. *Id.*, 209 U.S.P.Q. (BNA) at 51. Moreover, this argument may be construed as a tacit admission that the parent imidazole compound does possess the particular pharmacological activity of inhibiting thromboxane synthetase.

Along this line, we note that Dr. Smith, Cross' expert witness, testified generally, based upon the exhibits proffered by Iizuka, *see infra* note 18, that the parent imidazole compound possessed pharmacological activity for inhibiting thromboxane synthetase, although stating that

there was a wide potency spectrum for prior art imidazole derivatives with respect to the parent imidazole compound.

Cross has directed the court's attention to the fact that the Japanese priority application, while disclosing that the parent imidazole and 1-methylimidazole compounds possess an inhibitory action for thromboxane synthetase, further discloses that this inhibitory effect is not satisfactory and that the parent imidazole and 1-methylimidazole compounds have not been put to practical therapeutic use. But a therapeutical utility is not necessarily synonymous to a pharmacological activity. *Cf. Nelson*, 626 F.2d at 856, 206 U.S.P.Q. (BNA) at 883.

[**27] [*1049] The expert of Iizuka, Dr. Ramwell, testified that, as of the critical date, there was an awareness on the part of those skilled in the art that the parent imidazole compound exhibited an inhibitory activity for thromboxane synthetase, in both *in vitro* and *in vivo* environments. Dr. Ramwell further testified that there was an awareness by those skilled in the art of a correlation between thromboxane A[2] and platelet aggregation, namely that thromboxane A[2] was a mediator in platelet aggregation. Several exhibits proffered by Iizuka corroborated Dr. Ramwell's testimony as to the general knowledge in the art with respect to the inhibitory effect of the parent imidazole compound for thromboxane synthetase. [18] Accordingly, the similar pharmacological activity of the parent imidazole and 1-methylimidazole compounds have probative value in the factual determination of practical utility for the compounds of the phantom count inasmuch as Cross has not met the burden of proof to establish structural dissimilarities between the parent imidazole and 1-methylimidazole compounds and the imidazole derivatives of the phantom count. *Rey-Bellet*, 493 F.2d at 1386-87, [**28] 181 U.S.P.Q. (BNA) at 457.

18 For example, Table I in the article "*Imidazole*: A Selective Inhibitor of Thromboxane Synthetase," PROSTAGLANDINS, Vol. 13, No. 4, April 1977 (Iizuka Exhibit No. 6), lists 1-methylimidazole and the parent imidazole compounds as possessing inhibitory activity for thromboxane synthetase, thereby offering corroboration of Dr. Ramwell's testimony.

The Board noted that Iizuka Exhibits 2-6 and 10-12, while inadmissible for the purpose of establishing the truth of what they say on their face, are admissible to bolster and support the testimony of Dr. Ramwell, as well as for the purpose of establishing what literature was available to the art at the critical time. Thus, for review purposes, we have examined these exhibits for their corroborating value with respect to Dr. Ramwell's testimony.

The Board found that there was adequate proof that the Japanese priority application disclosed a pharmacological activity for the compounds of the phantom count in inhibiting the action of [**29] thromboxane synthetase, similar to the pharmacological activity of the parent imidazole and 1-methylimidazole compounds which were found to possess an inhibitory action for thromboxane synthetase, this disclosed knowledge of the inhibitory action of the prior art compounds having been corroborated by testimony and documentary evidence. During the proceedings before the Board, the burden of proof rested upon Cross to show that the Japanese priority application was deficient. 37 C.F.R. § 1.257(a). On review, Cross bears the burden of proof to show that the Board erred in finding that the Japanese priority application had adequately disclosed a practical utility. Reviewing the relevant evidence presented to the Board as a whole, we are not persuaded that Cross has met this burden of proof.

[*1050] The final question we must address is whether the inhibitory activity for thromboxane synthetase in human or bovine platelet microsomes, i.e., an *in vitro* utility, is sufficient to comply with the practical utility requirement of § 101. Based upon the facts of this case, we are not persuaded that the Board erred in finding that the *in vitro* utility disclosed in the Japanese [**30] priority application for the compounds of the count is sufficient to establish a practical utility.

Our predecessor court has noted that [HN9]adequate proof of any pharmacological activity constitutes a showing of practical utility. *See, e.g., Nelson*, 626 F.2d at 856, 206 U.S.P.Q. (BNA) at 883; *Rey-Bellet*, 493 F.2d at 1383, 181 U.S.P.Q. (BNA) at 454. Dr. Ramwell testified that initial testing of compounds for a particular pharmacological activity is typically done in vitro. In vitro testing permits an investigator to establish the rank order of compounds with respect to the particular pharmacological activity, i.e., to determine the relative potency of the compounds. Compounds having the highest ranking or potency are then selected for further testing *in vivo*. Presumably this is the accepted practice in the pharmaceutical industry inasmuch as Cross has not proffered any evidence refuting this testimony of Dr. Ramwell, and we note that this practice has an inherent logical persuasiveness. *In vitro* testing, in general, is relatively less complex, less time consuming, and less expensive than *in vivo* testing. Moreover, *in vitro* results with respect to the particular [**31] pharmacological activity are generally predictive of *in vivo* test results, i.e., there is a reasonable correlation therebetween. Were

this not so, the testing procedures of the pharmaceutical industry would not be as they are. Iizuka has not urged, and rightly so, that there is an invariable exact correlation between *in vitro* test results and *in vivo* test results. Rather, Iizuka's position is that successful *in vitro* testing for a particular pharmacological activity establishes a significant probability that *in vivo* testing for this particular pharmacological activity will be successful.

As discussed above, Dr. Ramwell testified that the parent imidazole and 1-methylimidazole compounds had been subjected to both *in vitro* and *in vivo* testing as of the critical date, this corroborated by documentary evidence, and found to possess an inhibitory effect for thromboxane synthetase. Based upon this, Dr. Ramwell further testified that he would expect that *in vivo* testing of the imidazole derivatives of the phantom count would show that these compounds also possessed an inhibitory action for thromboxane synthetase, i.e., there would be a reasonable correlation [**32] between *in vitro* test results and *in vivo* test results. This evidence was found sufficient by the Board as proof that the Japanese priority application had disclosed a completed practical utility for the imidazole derivatives of the phantom count in inhibiting thromboxane synthetase in human or bovine platelet microsomes. *Cf. Rey-Bellet*, 493 F.2d at 1386-87, 181 U.S.P.Q. (BNA) at 457.

Cross argues that the *in vitro* utility disclosed by the Japanese priority application is not *per se* useful, and that more sophisticated *in vitro* tests, using intact cells, or *in vivo* tests are necessary to establish a practical utility. [19] Cross is arguing that there must be a rigorous correlation of pharmacological activity between the disclosed *in vitro* utility and an *in vivo* utility to establish a practical utility. We, however, find ourselves in agreement with the Board that, based upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed *in vitro* utility and an *in vivo* activity, and therefore a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative [**33] evidence. *Cf. Nelson*, 626 F.2d at 856, 206 U.S.P.Q. (BNA) at 883-83.

19 Cross is seemingly arguing that the *in vitro* disclosure of the Japanese priority application is only a *potential* utility. *See Knapp v. Anderson*, 477 F.2d 588, 591, 177 U.S.P.Q. (BNA) 688, 691 (CCPA 1973).

Our predecessor court has accepted evidence of *in vivo* utility as sufficient to [*1051] establish a practical utility. *See, e.g., Nelson v. Bowler*, 626 F.2d 853, 206 U.S.P.Q. (BNA) 881 (CCPA 1980); *In re Jolles*, 628 F.2d 1322, 206 U.S.P.Q. (BNA) 885 (CCPA 1980); *Rey-Bellet v. Engelhardt*, 493 F.2d 1380, 181 U.S.P.Q. (BNA) 453 (CCPA 1974).

Opinions of our predecessor court have recognized the fact that pharmacological testing of animals is a screening procedure for testing new drugs for practical utility. *See, e.g., In re Jolles*, 628 F.2d 1322, 1327, 206 U.S.P.Q. (BNA) 885, 890 (CCPA 1980). This *in vivo* testing is but an intermediate link in a screening chain which may eventually lead to [**34] the use of the drug as a therapeutic agent in humans. We perceive no insurmountable difficulty, under appropriate circumstances, in finding that the first link in the screening chain, *in vitro* testing, may establish a practical utility for the compound in question. Successful *in vitro* testing will marshal resources and direct the expenditure of effort to further *in vivo* testing of the most potent compounds, thereby providing an immediate benefit to the public, analogous to the benefit provided by the showing of an *in vivo* utility. *Cf. Nelson*, 626 F.2d at 856, 206 U.S.P.Q. (BNA) at 883.

Today, under the circumstances of the instant case, where the Japanese priority application discloses an *in vitro* utility, i.e., the inhibition of thromboxane synthetase in human or bovine platelet microsomes, and where the disclosed *in vitro* utility is supplemented by the similar *in vitro* and *in vivo* pharmacological activity of structurally similar compounds, i.e., the parent imidazole and 1-methylimidazole compounds, we agree with the Board that this *in vitro* utility is sufficient to comply with the practical utility requirement of § 101.

3. Enablement

[**35] The Board found that the knowledge as to the use of the pharmacological activity disclosed in the Japanese priority application lay in the fact that the system was a microsome system, microsome systems admittedly being known to those skilled in the art. Employing a microsome assay, the skilled worker could determine the relative strength of the compounds of the count vis-a-vis the known parent imidazole and 1-methylimidazole compounds. Thus, the dosage in the microsome assay milieu could be determined without inventive skill or undue experimentation.

Since we have agreed with the Board that the practical utility for the imidazole derivatives of the phantom count lies in their pharmacological activity in the microsome environment, the how-to-use requirement of § 112 must be analyzed with reference to the microsome environment. We are confronted with a disclosure, similar to the situation before the court in *Bundy*, that fails to reveal dosages for the novel compounds *per se*. 642 F.2d at 434, 209 U.S.P.Q. (BNA) at 51. Although the Japanese priority application does disclose the fact that the imidazole derivatives of

the phantom count possess a pharmacological activity [**36] similar to the parent imidazole and 1-methylimidazole compounds, the priority application, unlike the application in *Bundy*, does not disclose dosages for the parent imidazole and 1-methylimidazole compounds.

We agree with the Board, however, that this deficiency in the Japanese priority application is not fatal. The testimonial evidence of Dr. Ramwell, corroborated by certain documentary evidence, showed that those skilled in the art had available, at the critical date, information as to approximate dosage levels for the parent imidazole and 1-methylimidazole compounds to produce an I[C50] effect, i.e., a 50% inhibition of thromboxane synthetase, in a microsome milieu. The objective of the pharmaceutical research undertaken by the parties was to discover imidazole derivatives having a potent inhibitory effect for thromboxane synthetase. Therefore, we believe it is logical, as did the Board, that the starting point for determining I[C50] dosage levels for the imidazole derivatives of the phantom count would be the I[C50] dosage levels of the parent imidazole and 1-methylimidazole compounds. The Board found that there was sufficient credible evidence that one skilled [**37] in the art, without the exercise of [*1052] inventive skill or undue experimentation, could determine the I[C50] dosage level for the imidazole derivatives of the phantom count in the microsome environment. *Cf. Bundy, id.*, 209 U.S.P.Q. (BNA) at 51. We do not believe the Board erred in arriving at this conclusion. This is not a case such as *In re Gardner*, 57 C.C.P.A. 1207, 427 F.2d 786, 166 U.S.P.Q. (BNA) 138 (1970), where the CCPA held that the applicant's disclosure was nonenabling because inventive skill and undue experimentation would be required to discover appropriate dosages for humans, i.e., a therapeutic use. In the instant case, we are confronted with a pharmacological activity or practical utility, not a therapeutic use.

While we agree with the Board that the disclosure in the Japanese priority application is somewhat confusing with respect to the 2.5 x 10<-8> level of molar concentrations, and that the 2-[p-(1-imidazolylmethyl) phenoxy]-acetic acid hydrochloride compound is outside the phantom count of the interference, this disclosed molar concentration, we believe, does provide some probative value going towards the sufficiency of the Japanese priority [**38] application for an enabling disclosure. The disclosed molar concentration would provide sufficient information as to an initial dosage level so that one skilled in the art could determine, without inventive skill or undue experimentation, the necessary molar concentrations for the imidazole derivatives of the phantom count to achieve the desired pharmacological effect, i.e., the 50% inhibition of thromboxane synthetase in human or bovine platelet microsomes.

The Board held the disclosure of the Japanese priority application adequate to satisfy the first paragraph of § 112. The burden is on Cross to show Board error in arriving at this conclusion, and we are not persuaded that Cross has successfully carried this burden. Accordingly, we are satisfied that the how-to-use requirement of § 112 has been complied with by the disclosures of the Japanese priority application.

AFFIRMED.

LEXSEE

Caution
As of: Aug 19, 2008

ATLAS POWDER COMPANY, Appellee, v. E.I. DU PONT DE NEMOURS & COMPANY AND ALAMO EXPLOSIVES COMPANY, INC., Appellants

No. 84-504

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

750 F.2d 1569; 1984 U.S. App. LEXIS 15324; 224 U.S.P.Q. (BNA) 409

December 27, 1984

SUBSEQUENT HISTORY: [**1] As Amended January 31, 1985.

PRIOR HISTORY: Appealed from: U.S. District Court for Texas.

DISPOSITION: AFFIRMED.

CASE SUMMARY:

PROCEDURAL POSTURE: Appellants sought review of a judgment by the United States District Court for the Northern District of Texas holding appellee's patent claims not invalid under 35 U.S.C.S. §§ 102, 103, and 112, not fraudulently procured, and infringed.

OVERVIEW: Appellee complained that appellants had infringed the patent that the inventor had assigned to appellee. The lower court held for appellee, and the court affirmed. The lower court's finding of no anticipation under 35 U.S.C.S. § 102 was not clearly erroneous, and its conclusion of nonobviousness under 35 U.S.C.S. § 103 was justified because at the time of patent's filing the patent's solution to problems would not have been obvious. In accordance with 35 U.S.C.S. § 112, appellee's patent enabled one skilled in the art to make the claimed invention. Even if some of the claimed combinations were inoperative, appellee's claims were not necessarily invalid, and the use of prophetic examples did not make the patent non-enabling. Appellee did not engage in inequitable conduct because its examples conformed to the Patent Office's requirements on prophetic examples. Although there was no literal infringement, appellants' product was equivalent to appellee's claimed invention.

OUTCOME: The court affirmed because appellee's patent claims were not invalid for anticipation, obviousness, or enablement; there was no inequitable conduct before the Patent Office; and appellee's claims were infringed under the doctrine of equivalents.

LexisNexis(R) Headnotes

Civil Procedure > Appeals > Standards of Review > Clearly Erroneous Review
Criminal Law & Procedure > Appeals > Standards of Review > General Overview
Patent Law > Jurisdiction & Review > Standards of Review > General Overview
[HN1]The burden is on appellant to establish that the district court's ultimate fact findings were clearly erroneous, that the district court's legal conclusions were erroneous, or that the findings underlying the ultimate findings or conclusions were clearly erroneous. The "clearly erroneous" standard is satisfied if the appellate court is left with the firm conviction that error has been committed.

Evidence > Procedural Considerations > Burdens of Proof > Clear & Convincing Proof
Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview

Patent Law > Infringement Actions > Defenses > Patent Invalidity > Validity Presumption
[HN2]Under 35 U.S.C.S. § 282, a patent is presumed valid, and the one attacking validity has the burden of proving invalidity by clear and convincing evidence.

Evidence > Inferences & Presumptions > Presumptions
Patent Law > Anticipation & Novelty > General Overview
Patent Law > Infringement Actions > Defenses > Patent Invalidity > Validity Presumption
[HN3]Though the introduction of prior art not before the Patent and Trademark Office may facilitate meeting the challenger's ability to meet the burden of proof on invalidity, the presumption remains intact, the burden of persuasion remains on the challenger, and the "clear and convincing" standard does not change.

Patent Law > Anticipation & Novelty > General Overview
[HN4]The exclusion of a claimed element from a prior art reference is enough to negate anticipation by that reference.

Patent Law > Nonobviousness > Elements & Tests > Claimed Invention as a Whole
Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN5]Though an invention is not anticipated by 35 U.S.C.S. § 102, a patent should not issue if the differences between the claimed invention and prior art are such that the invention as a whole would have been obvious to one of ordinary skill in the art at the time the invention was made.

Patent Law > Anticipation & Novelty > General Overview
Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Nonobviousness > Elements & Tests > Hindsight
[HN6]In assessing nonobviousness a court should answer certain factual inquiries: (1) the scope and content of the prior art; (2) the level of ordinary skill in the art; (3) the differences between the claimed invention and the prior art; and (4) so-called "secondary" considerations, e.g., long felt need, unexpected results, commercial success. The "secondary" considerations, when present, may assist the court in determining nonobviousness without falling prey to hindsight reasoning.

Patent Law > Claims & Specifications > Enablement Requirement > Standards & Tests
[HN7]To be enabling under 35 U.S.C.S. § 112, a patent must contain a description that enables one skilled in the art to make and use the claimed invention. That some experimentation is necessary does not preclude enablement; the amount of experimentation, however, must not be unduly extensive.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN8]Determining enablement is a question of law.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN9]It is not a function of the claims to specifically exclude possible inoperative substances. If the number of inoperative combinations becomes significant, and in effect forces one of ordinary skill in the art to experiment unduly in order to practice the claimed invention, the claims might be invalid.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Statutory Bars > Experimental Use > Elements
[HN10]Use of prophetic examples does not automatically make a patent non-enabling. The burden is on one challenging validity to show by clear and convincing evidence that the prophetic examples together with other parts of the specification are not enabling.

Patent Law > Inequitable Conduct > General Overview
[HN11]"Inequitable conduct" in the Patent and Trademark Office is a more appropriate label than "fraud."

Patent Law > Inequitable Conduct > Burdens of Proof
Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > U.S. Patent & Trademark Office Proceedings > General Overview
[HN12]Inequitable conduct requires proof by clear and convincing evidence of a threshold degree of materiality of the nondisclosed or false information. That threshold

can be established by any of four tests: (1) objective "but for"; (2) subjective "but for"; (3) "but it may have been"; and (4) 37 C.F.R. § 1.56(a), i.e., whether there is a substantial likelihood that a reasonable examiner would have considered the omitted or false information important in deciding whether to allow the application to issue as a patent. The Patent and Trademark Office (PTO) standard is the appropriate starting point because it is the broadest and most closely aligns with how one ought to conduct business with the PTO.

Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > Duties
Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > Fact & Law Issues
Torts > Negligence > Proof > Evidence > Inferences & Presumptions
[HN13]Inequitable conduct requires proof of a threshold intent. That intent need not be proven with direct evidence. It may be proven by showing acts the natural consequences of which are presumably intended by the actor. Proof of deliberate scheming is not needed; gross negligence is sufficient. Gross negligence is present when the actor knew or should have known of the materiality of a withheld reference. On the other hand, simple negligence, oversight or an erroneous judgment made in good faith is insufficient. Once the thresholds of materiality and intent are established as facts, the court must balance them and determine as a matter of law whether the scales compel a conclusion that inequitable conduct occurred. If the court reaches that conclusion, it must hold the patent claims at issue unenforceable.

Patent Law > Infringement Actions > Claim Interpretation > General Overview
Patent Law > Infringement Actions > Infringing Acts > General Overview
[HN14]Determining infringement requires claim construction as a preliminary step. If properly construed claims read on the infringing product, there is literal infringement.

Patent Law > Infringement Actions > Doctrine of Equivalents > Elements > General Overview
[HN15]A product that does not literally infringe can infringe under the doctrine of equivalents. Designed to protect a patentee from an infringer who appropriates the invention but avoids the literal language of the claims, the doctrine allows a finding of infringement when the accused product and claimed invention perform substantially the same function in substantially the same way to yield substantially the same result.

Patent Law > Infringement Actions > Doctrine of Equivalents > General Overview
[HN16]The doctrine of equivalence should not be the prisoner of a rigid formula. Consideration must be given to the purpose for which an ingredient is used in a patent, the qualities it has when combined with the other ingredients, and the function which it is intended to perform.

Patent Law > Infringement Actions > Doctrine of Equivalents > General Overview
[HN17]Where the accused product avoids literal infringement by changing one ingredient of a claimed composition, it is appropriate for a court to consider in assessing equivalence whether the changed ingredient has the same purpose, quality, and function as the claimed ingredient. If it does, the accused and claimed products should meet the Graver Tank tripartite test of "function, way, and result."

Patent Law > Infringement Actions > Doctrine of Equivalents > General Overview
[HN18]Where the defendant has appropriated the material features of the patent in suit, infringement will be found even when those features have been supplemented and modified to such an extent that the defendant may be entitled to a patent for the improvement.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Infringement Actions > Exclusive Rights > Manufacture, Sale & Use
Patent Law > Ownership > Conveyances > Licenses
[HN19]A patent is not the grant of a right to make or use or sell. It does not, directly or indirectly, imply any such right. It grants only the right to exclude others. The supposition that a right to make is created by the patent grant is obviously inconsistent with the established distinctions between generic and specific patents, and with the well-known fact that a very considerable portion of the patents granted are in a field covered by a former relatively generic or basic patent, are tributary to such earlier patent, and cannot be practiced unless by license thereunder.

Patent Law > U.S. Patent & Trademark Office Proceedings > Examinations > General Overview

[HN20]In examining a second application, the Patent Office has no concern with the scope of the claim of the first, and does not and must not pay any attention thereto. It is concerned only with the early disclosure by the specification and drawings.

Patent Law > Infringement Actions > Defenses > General Overview
[HN21]Patentable difference does not of itself tend to negative infringement. It may just as well be based upon infringement, plus improvement; and improvement may lie in addition, simplification, or variance.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Infringement Actions > Doctrine of Equivalents > Elements > Time of Equivalence
Patent Law > Infringement Actions > Doctrine of Equivalents > Improvements & New Equivalents
[HN22]It is not a requirement of equivalence that those skilled in the art know of the equivalence when the patent application is filed or the patent issues. That question is determined as of the time infringement takes place. Devices changing the patented invention with advances developed subsequent to the patent can infringe under the doctrine of equivalents.

Patent Law > Infringement Actions > Doctrine of Equivalents > General Overview
[HN23]Finding equivalence is not inconsistent with a patentee's unsuccessful attempt to make the accused product. The focus in assessing equivalence is on whether the accused product performs substantially the same as the claimed product in function, way and result -- it is not on the patentee's ability to devise a product equivalent to the patented product. Indeed, the patentee's incentive to devise an equivalent product is often less than a competitor's, which alone may account for the competitor's success and the patentee's failure in devising the equivalent product.

Civil Procedure > Appeals > Reviewability > Preservation for Review
[HN24]A party may generally not argue on appeal an issue not raised below.

Patent Law > Infringement Actions > Doctrine of Equivalents > General Overview
[HN25]Although there is no legally recognized "essence" or "heart" of the invention in determining validity, it can be applicable in a determination of infringement under the doctrine of equivalents.

Civil Procedure > Judgments > General Overview
Civil Procedure > Appeals > Reviewability > Adverse Determinations
[HN26]The appellate court reviews judgments, not statements in opinions.

COUNSEL: Lawrence F. Scinto, Fitzpatrick, Cella, Harper & Scinto, of New York, New York, argued for Appellants. With him on the brief were Joseph M. Fitzpatrick, David F. Ryan, Nels T. Lippert and Laura A. Bauer, of Counsel.

Robert C. Kline and James R. Morrison, of Wilminton, Delaware and Stanley E. Neely, Locke, Purnell, Boren, Laney & Neely, of Dallas, Texas, were on the brief for Appellant.

Garland P. Andrews, Richards, Harris & Medlock, of Dallas, Texas, argued for Appellee. With him on the brief were Roy W. Hardin and David L. Hitchcock.

JUDGES: Markey, Chief Judge, Baldwin, and Miller, Circuit Judges.

OPINION BY: BALDWIN

OPINION

[*1571] BALDWIN, Circuit Judge.

This is an appeal by E.I. du Pont De Nemours & Co. and its customer Alamo Explosives Co., Inc. (collectively, "Du Pont"). The appeal is from a final judgment of the United States District Court for the Northern District of Texas holding product claims 1-5, 7, 12-14, and 16-17 of U.S. Patent No. 3,447,978 ('978 patent), issued to Harold Bluhm on June 3, 1969 and assigned to the Atlas Powder Co. ("Atlas"), not invalid under [**2] 35 U.S.C. §§ 102, 103, and 112, not fraudulently procured, and infringed. We affirm.

Background

The district court opinion, reported at 588 F. Supp. 1455, 221 U.S.P.Q. (BNA) 426 (1983), contains a detailed description of the facts, familiarity of which is presumed herein.

Briefly, the '978 patent relates to blasting agents, *i.e.*, chemical mixtures that are relatively insensitive to normal modes of detonation but can be made to detonate with a high strength explosive primer. By the mid-1960's, blasting agents consisted of two major types: "ANFO" and "water-containing".

An "ANFO" blasting agent comprised a mixture of particulate ammonium nitrate, usually in the form of small round aggregates known as "prills", and fuel oil (*e.g.*, diesel fuel). They were widely used in mining and construction because of their low cost, ease of handling, and ability to be mixed at the blast site rather than prepackaged at the plant. However, to work properly they could be used only in "dry" holes (without water) because water desensitized the mixture, rendering it nondetonable.

A "water-containing" blasting agent, which was water resistant, generally comprised a slurry of particulate [**3] ammonium nitrate (or other oxidizing salt), a solid or liquid fuel, at least 5 percent water, and, as a sensitizer to increase explosive power, either a high explosive such as TNT or a chemical such as nitric acid. Often, a gelling agent was added, particularly in the chemical sensitized slurries, to prevent the separation of sensitizers from slurry by [*1572] forming a gel (a colloid in which the disperse phase has combined with the continuous phase to produce a viscous, jelly-like product). The use of sensitizers in water-containing blasting agents made preparation and handling more difficult and dangerous and, hence, more costly.

Before the '978 invention, Atlas manufactured a gelled slurry blasting agent called Aquanite, based on U.S. Patent No. 3,164,503, issued to Gehrig and assigned to Atlas. Aquanite used as a sensitizer nitric acid, which was highly caustic to skin and clothing and tended to separate out of the product even in the presence of a gelling agent, thereby reducing the product's stability and shelf life. Also, Aquanite was "hypergolic", *i.e.*, it ignited wood, coal and various chemicals upon contact, which was suspected of causing the blasting agent [**4] to detonate prematurely.

The Invention

In 1965, Atlas assigned Harold Bluhm to investigate stabilizing its Aquanite gel. Bluhm experimented with various "emulsions" that did not contain nitric acid or a gelling agent. (An emulsion is a stable mixture of two immiscible liquids; a "water-in-oil" emulsion has a continuous oil and discontinuous aqueous phase; an "oil-in-water" emulsion is the reverse.) In early 1966, Bluhm formulated an intimately mixed water-in-oil, water resistant emulsion blasting agent. The product was sensitized with entrapped air rather than high explosives or chemicals and is the subject matter of the claims at issue. Representative is Claim 1:

1. An emulsion blasting agent consisting essentially of:

an aqueous solution of ammonium nitrate forming a discontinuous emulsion phase;

a carbonaceous fuel forming a continuous emulsion phase;

an occluded gas dispersed within said emulsion and comprising at least 4% by volume, thereof at 70 degrees F. and atmospheric pressure; and

a water-in-oil type emulsifying agent;

said carbonaceous fuel having a consistency such that said occluded gas is held in said emulsion at a temperature of 70 degrees [**5] F.

Claim 1 is the only independent claim in suit. The other, dependent claims describe various ingredients, such as microspheres for the occluded gas, additional fuels (*e.g.*, aluminum), specific ranges of ingredients, and various properties of the blasting agent.

Du Pont's Activities

Du Pont sold a gelled slurry blasting agent until the latter part of the 1970's. In 1976, Du Pont formed a team to study the feasibility of an emulsion blasting agent. The team succeeded in making a water-in-oil emulsion blasting agent which Du Pont began making and selling in August 1978. Atlas sued for infringement in December 1979.

The District Court Proceedings

A non-jury trial was held between January 28 and February 2, 1982. Du Pont asserted invalidity of the '978 patent under sections 102(a), 103, and 112, "fraud" on the Patent and Trademark Office (PTO), and noninfringement. The district court rejected those assertions for the product claims at issue, holding that: (1) the claimed invention was not anticipated by the prior art; (2) the claimed invention would not have been obvious in view of the prior art; (3) the claims were not invalid for the patent's failure [**6] to comply with the "best mode", enablement, and "overclaiming" requirements of 35 U.S.C. § 112; (4) the patent was not procured by "fraud" on the PTO; and (5) Du Pont's products infringed the claims under the doctrine of equivalence. On appeal, Du Pont contests those holdings, except for the one on best mode.

The district court denied Atlas increased damages and attorney fees because Du Pont had not willfully infringed the '978 patent claims and the case was not "exceptional". The district court also held that product claims 6, 13, and 15 were not infringed and that process

claims 18-30 were invalid. Atlas has not appealed those holdings.

[*1573] *Issues*

(1) Whether the district court was clearly erroneous in finding the invention of the patent claims at issue not anticipated by the prior art.

(2) Whether the district court erred in holding that the invention of the patent claims at issue would not have been obvious.

(3) Whether the district court erred in holding the patent claims at issue not invalid because of nonenablement.

(4) Whether the district court erred in holding no "fraud" on the PTO, *i.e.*, no inequitable conduct.

(5) Whether the district [**7] court was clearly erroneous in finding that Du Pont's products infringed the '978 claims under the doctrine of equivalents.

OPINION

I. *Standard of Review*

[HN1]The burden is on Du Pont, as appellant, to establish that the district court's ultimate fact findings (*e.g.*, anticipation, infringement) were clearly erroneous, that the district court's legal conclusions (*e.g.*, § 103 obviousness, § 112 enablement) were erroneous, or that the findings underlying the ultimate findings or conclusions were clearly erroneous. The "clearly erroneous" standard is satisfied if we are left with the firm conviction that error has been committed. *See, e.g., Raytheon Co. v. Roper Corp.*, 724 F.2d 951, 956, 220 U.S.P.Q. (BNA) 592, 596 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835, 105 S. Ct. 127, 83 L. Ed. 2d 69, 225 U.S.P.Q. (BNA) 232, 53 U.S.L.W. 3237 (1984).

II. *Presumption of Validity*

[HN2]Under 35 U.S.C. § 282, a patent is presumed valid, and the one attacking validity has the burden of proving invalidity by clear and convincing evidence. *See, e.g., American Hoist & Derrick Co. v. Sowa & Sons, Inc.*, 725 F.2d 1350, 1360, 220 U.S.P.Q. (BNA) 763, 770 (Fed. Cir. 1984), [**8] *cert. denied*, 469 U.S. 821, 105 S. Ct. 95, 83 L. Ed. 2d 41, 53 U.S.L.W. 3236, 224 U.S.P.Q. (BNA) 520 (1984). In that regard, the district court committed an error.

After correctly stating that the presumption of validity must be overcome with clear and convincing evidence, the district court stated that, if pertinent prior art were not cited to the PTO, as was the case here, the presumption is weakened and Du Pont must prove invalidity by only a preponderance of the evidence. That is incorrect. [HN3]Though the introduction of prior art not before the PTO may facilitate meeting the challenger's ability to meet the burden of proof on invalidity, the presumption remains intact, the burden of persuasion remains on the challenger, and the "clear and convincing" standard does not change. *See, e.g., Jervis B. Webb Co. v. Southern Systems, Inc.*, 742 F.2d 1388, 1392 & n.4, 222 U.S.P.Q. (BNA) 943, 945 & n.4 (Fed. Cir. 1984); *Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 1534, 218 U.S.P.Q. (BNA) 871, 875 (Fed. Cir. 1983).

The error, however, was harmless. Indeed, it helped Du Pont at trial by lowering the standard of proof needed to prove its case. Even with the lower standard, [**9] Du Pont was unable to succeed.

III. *Anticipation*

The district court's determination of no anticipation was a factual one that should be reversed only if appellant shows that it was clearly erroneous. *See, e.g., Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1458, 221 U.S.P.Q. (BNA) 481, 485 (Fed. Cir. 1984). Du Pont attempts to satisfy its burden by arguing the U.S. Patent No. 3,161,551, to Egly, et al., anticipated the claimed invention. We conclude, however, that the district court's finding of no anticipation was not clearly erroneous.

Egly, which Du Pont referred to at oral argument as the "closest prior art", describes an emulsion of ammonium nitrate, water, fuel oil, and a water-in-oil emulsifying agent. Though Egly teaches the presence of solid ammonium nitrate prills as an essential ingredient, Du Pont argues that the '978 claims, because of the phrase "consisting essentially of", does not exclude the presence of those prills. *See, e.g., In re Herz*, 537 F.2d 549, 551, 190 U.S.P.Q. (BNA) 461, 463 (CCPA 1976); *In re Janakirama-Rao*, 50 C.C.P.A. 1312, 317 F.2d 951, 954, 137 U.S.P.Q. (BNA) 893, 896 [*1574] (1963). Du [**10] Pont is correct. However, the district court found that Egly "does not mention air or gas as an ingredient in their explosives" and occluded air is an element of the claims. Hence, there is no anticipation under § 102, because [HN4]the exclusion of a claimed element from a prior art reference is enough to negate anticipation by that reference. *Kalman v. Kimberly-Clark Corp.*, 713 F.2d 760, 771-72, 218 U.S.P.Q. (BNA) 781, 789 (Fed. Cir. 1983).

Du Pont asserts that Bluhm conceded in answer to an interrogatory that the first reduction to practice of the claimed invention was on January 14, 1966, and that Mr. Bluhm's notebook shows the composition prepared on that date to be identical to Egly's, *i.e.*, an emulsion without occluded air. Because the first reduction to practice was identical to Egly's product, Du Pont argues, the claimed invention is anticipated by Egly. Atlas

argues that the notebook entry reveals that occluded air was present in the composition prepared on January 14, 1966, and hence, the first reduction to practice was not identical to Egly's composition. Atlas appears to be correct but, in any event, the district court's anticipation analysis properly focused [**11] on the claimed invention, which includes occluded air, not on Atlas' characterization of the January 14, 1966 experiment as the first reduction to practice.

IV. *Obviousness*

[HN5]Though an invention is not anticipated by 35 U.S.C. § 102, a patent should not issue if the differences between the claimed invention and prior art are such that the invention as a whole would have been obvious to one of ordinary skill in the art at the time the invention was made. 35 U.S.C. § 103. [HN6]In assessing nonobviousness a court should answer certain factual inquiries: (1) the scope and content of the prior art; (2) the level of ordinary skill in the art; (3) the differences between the claimed invention and the prior art; and (4) so-called "secondary" considerations, *e.g.*, long felt need, unexpected results, commercial success. *See, e.g., Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d at 1538, 218 U.S.P.Q. (BNA) at 876; *Simmons Fastener Corp. v. Illinois Tool Works, Inc.*, 739 F.2d 1573, 1575, 222 U.S.P.Q. (BNA) 744, 746 (Fed. Cir. 1984). The "secondary" considerations, when present, may assist the court in determining nonobviousness without falling prey to hindsight reasoning.

Here, [**12] the district court made findings on the content of the prior art, the level of ordinary skill in the art, the differences between the prior art and the claimed invention as a whole, and then concluded that the claimed invention was nonobvious. Du Pont has not shown error in the legal conclusion of nonobviousness, or clear error in the underlying findings.

Content of the Prior Art and Differences Between It and the Claimed Invention

In addition to Egly, discussed above, the district court considered several patents and articles.

Atlas' Gehrig patent describes a blasting agent containing particulate ammonium nitrate, a solution of nitric acid in water, and fuel oil. Though the mixture may be an emulsion, the primary thrust of Gehrig is using a gel. Gehrig notes that, when an emulsion is used, the product quickly separates into its various components. Gehrig recommends that the emulsion be used within 24 hours to avoid separation. The gel form is considered desirable to stabilize the product for storage.

The claimed invention differs from Gehrig because Gehrig requires nitric acid as an essential ingredient. The '978 claims exclude the presence of nitric acid because [**13] the essence of the claimed composition is the elimination of nitric acid and the claim phrase "consisting essentially of" excludes ingredients that would "materially affect the basic and novel characteristics" of the claimed composition. *In re Herz*, 537 F.2d at 551, 190 U.S.P.Q. (BNA) at 463; *In re Janakirama-Rao*, 317 F.2d at 954, 137 U.S.P.Q. (BNA) at 895.

Gehrig does not teach or suggest substituting nitric acid with air to sensitize the product. Though it suggests the use of microballoons containing air as a stabilizer, [*1575] it also discusses heating the product to remove entrapped air.

U.S. Patent No. 3,052,578, to Davis, describes a blasting agent comprising a blend of fuel oil and ammonium nitrate poured over solid ammonium nitrate. An oil-in-water, not water-in-oil, emulsifying agent is suggested to disperse the fuel. Though an emulsifying agent is used for dispersing purposes, the reference does not discuss forming an emulsion, and it does not suggest use of occluded air.

Two papers by Coxon relate to water resistant blasting agents. The first describes a water-in-oil emulsion of fuel oil and ammonium nitrate poured over solid ammonium nitrate. The [**14] second is similar, but prefers an oil-in-water emulsifying agent. Neither paper teaches the presence of occluded air; instead, the blasting agent requires solid ammonium nitrate. Thus, both Coxon papers, as well as Davis, are similar to Egly.

U.S. Patent No. 3,004,842, to Rowlinson, describes melting solid ammonium nitrate and mixing it with fuel oil and an emulsifying agent to form a solid blasting agent. A small amount of water may be added to reduce the melting point of the ammonium nitrate. Foaming agents can be added to increase the product's sensitivity.

U.S. Patent No. 3,453,158, to Clay, describes a gel or thickened slurry containing aqueous ammonium nitrate, a gelling agent or thickener, air bubbles serving as a sensitizer, and particulate fuels or sensitizers. The district court found that Clay does not use an emulsion, let alone a water-in-oil emulsion, and that finding has not been shown to be clearly erroneous.

Level of Skill In The Art

The district court found that the person of ordinary skill in the art would be one skilled in the art of explosives formulation, having knowledge of and experience with the chemical and physical properties of explosives. [**15] The person should be a chemist or chemical engineer with at least a bachelor's degree and several years of practical experience. Also, he or she should have a working knowledge of the principles of emulsion chemistry as applied to explosives formulation.

"Secondary" Considerations

The district court stated that, in light of "substantial differences" between the prior art and the product claims, it is not necessary to consider secondary factors, though they were raised by Atlas. Hence, the district court's opinion does not contain a section on "secondary criteria" or otherwise attempt to identify such criteria under that label. Nevertheless, the district court found that "the Bluhm patent solved the problem of finding a water resistent ANFO blasting agent that did not require chemical sensitizers". Moreover, the district court in essence found that the solution to the problem was unexpected.

Though the prior art describes water-in-oil emulsions containing dissolved ammonium nitrate, fuel oil, and a water-in-oil emulsifying agent, the district court found that the art does not suggest that the emulsion itself can serve as a blasting agent. Egly, for example, teaches that [**16] such an emulsion -- without occluded air -- serves as a sensitizer that can be poured over solid ammonium nitrate to form a blasting agent. Gehrig teaches that the emulsion serves as a blasting agent only in the presence of nitric acid. That the Egly sensitizer itself serves as a blasting agent when occluded air is added, or that the Gehrig blasting agent could serve in that capacity without nitric acid, was unexpected. Though occluded air was recognized as an ingredient that could be included in blasting agent compositions, *e.g.*, to stabilize the nitric acid containing product of Gehrig, the district court found that the references simply did not teach "that aeration can substitute for chemical sensitizers [*e.g.*, nitric acid] in slurry explosives or that a water-in-oil emulsion is the most efficient system for entraining air".

Moreover, the district court found (and it has not been shown to be clearly erroneous) that the references cited by Du Pont de-emphasize occluded air in emulsions and, hence, teach away from the importance of aeration. Egly and Davis do not mention [*1576] air or gas as an ingredient in their explosives, and one of the Coxon papers teaches [**17] that detonation performance may be improved by using emulsifiers to eliminate frothing (air) from explosives.

Conclusion on Nonobviousness

In light of the differences between the claimed invention and prior art, the '978 solution to a troublesome problem, and the unexpected result that a water-in-oil emulsion of ammonium nitrate, fuel oil, and a water-in-oil emulsifying agent can serve as a blasting agent in the presence of occluded air, we agree with the district court's conclusion of nonobviousness.

Du Pont argues that it would have been obvious in 1966 to leave the nitric acid sensitizer out of Gehrig's slurry, intimately mix the fuel oil and ammonium nitrate, and sensitize the product in some other way, *e.g.*, with air. We agree with the district court, however, that neither Gehrig nor the other prior art suggests those changes to obtain an emulsion blasting agent. As stated by the district court:

> It is quite a leap from recognition that dry ANFOs could be sensitized by aeration to realization that if an ANFO slurry was placed in the proper form of a water-in-oil emulsion and aerated, it would not require chemical sensitizers for detonability. This leap [**18] would not have been obvious in 1966.

V. *Enablement*

The district court rejected Du Pont's arguments of "overly broad", "overclaiming", and "non-enablement", and its argument that the broad scope of the claims is not supported by the limited disclosure present. In essence, those arguments are one: the '978 disclosure does not enable one of ordinary skill in the art to make and use the claimed invention, and hence, the claimed invention is invalid under 35 U.S.C. § 112, para. 1.

[HN7]To be enabling under § 112, a patent must contain a description that enables one skilled in the art to make and use the claimed invention. *Raytheon Co. v. Roper Corp.*, 724 F.2d at 960, 220 U.S.P.Q. (BNA) at 599. That some experimentation is necessary does not preclude enablement; the amount of experimentation, however, must not be unduly extensive. *See, e.g., W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1557, 220 U.S.P.Q. (BNA) 303, 316 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851, 105 S. Ct. 172, 83 L. Ed. 2d 107, 53 U.S.L.W. 3239 (1984); *In re Angstadt*, 537 F.2d 498, 503, 190 U.S.P.Q. (BNA) 214, 218 (CCPA 1976). [HN8]Determining enablement is a question of [**19] law. *Raytheon Co. v. Roper Corp.*, 724 F.2d at 959-60, 220 U.S.P.Q. (BNA) at 599.

Du Pont argues that the patent disclosure lists numerous salts, fuels, and emulsifiers that could form thousands of emulsions but there is no commensurate teaching as to which combination would work. The disclosure, according to Du Pont, is nothing more than "a list of candidate ingredients" from which one skilled in the art would have to select and experiment unduly to find an operable emulsion.

The district court held it would have been impossible for Bluhm to list all operable emulsions and

exclude the inoperable ones. Further, it found such list unnecessary, because one skilled in the art would know how to select a salt and fuel and then apply "Bancroft's Rule" to determine the proper emulsifier. Bancroft's Rule was found by the district court to be a "basic principle of emulsion chemistry," and Du Pont has not shown that finding to be clearly erroneous.

We agree with the district court's conclusion on enablement. Even if some of the claimed combinations were inoperative, the claims are not necessarily invalid. [HN9]"It is not a function of the claims to specifically exclude . . . possible [**20] inoperative substances . . . ." *In re Dinh-Nguyen*, 492 F.2d 856, 858-59, 181 U.S.P.Q. (BNA) 46, 48 (CCPA 1974) (emphasis omitted). *Accord, In re Geerdes*, 491 F.2d 1260, 1265, 180 U.S.P.Q. (BNA) 789, 793 (CCPA 1974); *In re Anderson*, 471 F.2d 1237, 1242, 176 U.S.P.Q. (BNA) 331, 334-35 (CCPA 1973). Of course, if the number of inoperative combinations becomes significant, and in effect forces one of ordinary skill in the art to experiment unduly in order to practice the claimed invention, the [*1577] claims might indeed be invalid. *See, e.g., In re Cook*, 58 C.C.P.A. 1049, 439 F.2d 730, 735, 169 U.S.P.Q. (BNA) 298, 302 (1971). That, however, has not been shown to be the case here.

Du Pont contends that, because the '978 examples are "merely prophetic", they do not aid one skilled in the art in making the invention. [1] Because they are prophetic, argues Du Pont, there can be no guarantee that the examples would actually work.

1 The PTO Manual of Patent Examining Procedure (MPEP) § 608.01(p)(D) (5th ed. 1983), states:

> Simulated or predicted test results and prophetical examples (paper examples) are permitted in patent applications. Working examples correspond to work actually performed and may describe tests which have actually been conducted and results that were achieved. Paper examples describe the manner and process of making an embodiment of the invention which has not actually been conducted. Paper examples should not be represented as work actually done. Paper examples should not be described using the past tense.

[**21] [HN10]Use of prophetic examples, however, does not automatically make a patent non-enabling. The burden is on one challenging validity to show by clear and convincing evidence that the prophetic examples together with other parts of the specification are not enabling. Du Pont did not meet that burden here. To the contrary, the district court found that the "prophetic" examples of the specification were based on actual experiments that were slightly modified in the patent to reflect what the inventor believed to be optimum, and hence, they would be helpful in enabling someone to make the invention.

Du Pont argues that of some 300 experiments performed by Atlas before the filing of the '978 patent application, Atlas' records indicated that 40 percent failed "for some reason or another". The district court agreed that Atlas' records showed 40 percent "failed", but found that Atlas' listing of an experiment as a "failure" or "unsatisfactory" was misleading. Experiments were designated "failures", the district court found, in essence because they were not optimal under all conditions, but such optimality is not required for a valid patent. *Decca Ltd. v. United States*, 210 Ct. Cl. [**22] 546, 544 F.2d 1070, 1077, 191 U.S.P.Q. (BNA) 439, 444-45 (1976). *Accord, E.I. du Pont de Nemours & Co. v. Berkley & Co.*, 620 F.2d 1247, 1260, 205 U.S.P.Q. (BNA) 1, 10 (8th Cir. 1980). *Cf. Raytheon Co. v. Roper Co.*, 724 F.2d at 958, 220 U.S.P.Q. (BNA) at 598. The district court also found that one skilled in the art would know how to modify slightly many of those "failures" to form a better emulsion. Du Pont has not persuaded us that the district court was clearly erroneous in those findings.

Du Pont asserts that Atlas was able to produce suitable emulsions with only two emulsifiers, "Atmos 300" and "Span 80", and therefore, the disclosure should be construed to read upon only those two emulsifiers. However, Du Pont did not prove that the other disclosed emulsifiers were inoperable. The district court credited testimony by Atlas' expert, Dr. Fowkes, to the effect that he had successfully formed a number of detonable emulsions using a variety of emulsifiers specified in the '978 patent. Further, the district court found that one skilled in the art would know which emulsifiers would work in a given system. Indeed, the district court found that Du Pont's own researchers had [**23] little difficulty in making satisfactory emulsions with the emulsifying agents, salts, and fuels listed in the '978 patent. Those findings have not been shown to be clearly erroneous.

In sum, we conclude that Du Pont has failed to show that the district court erred in determining enablement.

VI. *Inequitable Conduct*

This court has held "inequitable [HN11]conduct" in the PTO to be a more appropriate label than "fraud". *J.P. Stevens & Co. v. Lex Tex Ltd.*, 747 F.2d 1553 (Fed. Cir. 1984). Hence, this opinion will use the phrase "inequitable conduct" rather than "fraud".

[HN12]Inequitable conduct requires proof by clear and convincing evidence of a threshold degree of materiality of the nondisclosed [*1578] or false information. That threshold can be established by any of four tests: (1) objective "but for"; (2) subjective "but for"; (3) "but it may have been"; and (4) 37 C.F.R. § 1.56(a), *i.e.*, whether there is a substantial likelihood that a reasonable examiner would have considered the omitted or false information important in deciding whether to allow the application to issue as a patent. Slip op. at 10. The PTO standard is the appropriate starting point because it [**24] is the broadest and most closely aligns with how one ought to conduct business with the PTO. *Id.*

[HN13]Inequitable conduct also requires proof of a threshold intent. That intent need not be proven with direct evidence. It may be proven by showing acts the natural consequences of which are presumably intended by the actor. *Id.* Proof of deliberate scheming is not needed; gross negligence is sufficient. Gross negligence is present when the actor knew or should have known of the materiality of a withheld reference. *Id.* at 11. On the other hand, simple negligence, oversight or an erroneous judgment made in good faith is insufficient. *Id.*

Once the thresholds of materiality and intent are established as facts, the court must balance them and determine as a matter of law whether the scales compel a conclusion that inequitable conduct occurred. *Id.* If the court reaches that conclusion, it must hold the patent claims at issue unenforceable.

Du Pont argues that Atlas committed inequitable conduct by failing to tell the examiner that the examples were "prophetic" and, hence, in misleading the examiner into believing that the examples were actually performed. However, [**25] the district court found that the examples were written in the present tense to conform with the PTO requirements on prophetic examples. Moreover, the district court found that all but one of the examples were based on actual experiments and only slightly modified to reflect the inventor's notion of the most effective formulation. Consequently, the district court found, there was no intent on the part of Atlas to mislead the PTO. Du Pont has not shown those findings to be clearly erroneous. Even if intent could be inferred, and if the examples were prophetic but not disclosed to the examiner as such, Du Pont has not shown the nondisclosure to have been material, *i.e.*, important to an examiner in allowing the patent to issue.

Du Pont asserts that Atlas' conduct cannot be distinguished from that in *Grefco, Inc. v. Kewanee Industries, Inc.*, 499 F. Supp. 844 (D. Del. 1980), *aff'd without publ. opinion*, 671 F.2d 495 (3d Cir. 1981). We disagree. In *Grefco*, the patentee, to convince the examiner of the invention's superiority, presented "test results" based on tests that it knew never occurred, told the examiner the invention had been successfully tested when it had [**26] twice failed, and withheld information about those failures from the examiner. Intent and materiality were clearly established in *Grefco*, and the court in weighing the two factors held that there was inequitable conduct. That is not true here.

Du Pont argues that Atlas did not disclose its numerous "failures" and that it "padded" the disclosure with emulsifiers it knew would not work. The district court, however, found that Du Pont failed to prove that any of the emulsifiers were inoperative and the court found that the evidence on the "failed" experiments was not dispositive. Du Pont has not shown any clear error on the part of the district court in those findings.

Du Pont also alleges inequitable conduct in Atlas not disclosing to the examiner its Aquanite gel, the commercial version of the invention of its Gehrig patent. Though the district court found Aquanite to be "pertinent", it found no intent in the nondisclosure because Atlas had disclosed the Gehrig patent to the examiner. Du Pont has not shown any clear error in that finding. *Cf. Vandenberg v. Dairy Equipment Co.*, 740 F.2d 1560, 1568-69, 224 U.S.P.Q. (BNA) 195, (Fed. Cir. 1984). (Vandenberg disclosed [**27] a PX-15 device as prior art but failed to describe it as its own prior invention; the disclosure was held to [*1579] be inconsistent with the intent necessary for inequitable conduct).

VII. *Infringement*

Literal Infringement

[HN14]Determining infringement requires claim construction as a preliminary step. *See, e.g., Fromson v. Advance Offset Plate, Inc.*, 720 F.2d 1565, 1569, 219 U.S.P.Q. (BNA) 1137, 1140 (Fed. Cir. 1983). If properly construed claims read on the infringing product, there is literal infringement. *Id.* at 1571, 219 U.S.P.Q. (BNA) at 1142.

Du Pont's blasting agents are water-in-oil emulsions containing water, ammonium nitrate, fuel oil, occluded gas, and an emulsifying agent. Unlike the claimed invention, Du Pont uses as the emulsifying agent sodium oleate, which is formed *in situ* by adding sodium hydroxide and oleic acid to the other emulsion ingredients. Sodium oleate is normally an oil-in-water emulsifying agent but in the environment of the Du Pont

product (*i.e.*, a high salt concentration leading to phase inversion), the sodium oleate acts as a water-in-oil emulsifying agent. The Du Pont product, and the *in situ* process of [**28] forming it, are the subject of U.S. Patent No. 4,287,100, issued to Owen and assigned to Du Pont.

The district court construed the '978 claim term "water-in-oil type emulsifying agent" as excluding compounds that normally function as oil-in-water emulsifying agents, *e.g.*, sodium oleate. That claim construction prompted the district court to find no literal infringement. Atlas does not contest that finding and, for purposes of appeal, we accept it and the underlying claim construction.

Doctrine of Equivalents

[HN15]A product that does not literally infringe can infringe under the doctrine of equivalents. Designed to protect a patentee from an infringer who appropriates the invention but avoids the literal language of the claims, the doctrine allows a finding of infringement when the accused product and claimed invention perform substantially the same function in substantially the same way to yield substantially the same result. *Graver Tank & Mfg. Co. v. Linde Air Products Co.*, 339 U.S. 605, 608-09, 94 L. Ed. 1097, 70 S. Ct. 854 (1950); *Perkin-Elmer Corp. v. Computervision Corp.*, 732 F.2d 888, 900, 221 U.S.P.Q. (BNA) 669, 679 (Fed. Cir. 1984), *cert. denied*, 469 [**29] U.S. 857, 105 S. Ct. 187, 83 L. Ed. 2d 120, 53 U.S.L.W. 3239, 225 U.S.P.Q. (BNA) 792 (1984). The district court found that the Du Pont products and the claimed invention are equivalent, and Du Pont has not shown that finding to be clearly erroneous. [2]

> 2 One of Du Pont's products includes aluminum, which is not present in representative claim 1. It is, however, present in dependent claim 14. Moreover, the addition of an ingredient by Du Pont does not necessarily avoid infringement of claim 1. *See, e.g., Radio Steel & Mfg. Co. v. MTD Products, Inc.*, 731 F.2d 840, 848, 221 U.S.P.Q. (BNA) 657, 663-64 (Fed. Cir. 1984), *cert. denied*, 469 U.S. 831, 105 S. Ct. 119, 83 L. Ed. 2d 62, 53 U.S.L.W. 3237 (1984); *Amstar Corp. v. Envirotech Corp.*, 730 F.2d 1476, 1482, 221 U.S.P.Q. (BNA) 649, 653 (Fed. Cir. 1984).

The district court's opinion clearly delineates the *Graver Tank* tripartite test of substantially the same function, way, and result but then, adopting an analysis found in *Ziegler v. Phillips* [**30] *Petroleum Co.*, 483 F.2d 858, 870, 177 U.S.P.Q. (BNA) 481, 487 (5th Cir.), *cert. denied*, 414 U.S. 1079, 38 L. Ed. 2d 485, 94 S. Ct. 597, 180 U.S.P.Q. (BNA) 1 (1973), focuses on the "function, purpose, and quality" of the emulsifying agents of Du Pont and the claimed invention. That focus, argues Du Pont, was wrong because it ignored the *Graver Tank* tripartite test. We disagree.

Though *Graver Tank* articulates the tripartite test of "function, way, and result", it also states that [HN16]the doctrine of equivalence should not be the prisoner of a rigid formula. Moreover, *Graver*, which as here compared a claimed mixture with an accused mixture in which one ingredient of the claimed mixture was changed, stated:

> "Consideration must be given to the purpose for which an ingredient is used in a patent, the qualities it has when combined with the other ingredients, and the function which it is intended to perform."

Id. at 611, 70 S. Ct. at 857.

Such consideration makes sense. [HN17]Where, as here, the accused product avoids literal infringement by changing one ingredient [*1580] of a claimed composition, it is appropriate for a court to consider in assessing [**31] equivalence whether the changed ingredient has the same purpose, quality, and function as the claimed ingredient. If it does, the accused and claimed products should meet the *Graver Tank* tripartite test of "function, way, and result".

That the district court focused on the function, quality, and purpose of the emulsifying agents does not mean it ignored the basic tripartite test which it expressly referred to in the opinion. We infer from that express reference, and from the opinion as a whole, that the district court did in fact find that the "function, way, and result" test was satisfied. *See ACS Systems, Inc. v. Montefiore Hospital*, 732 F.2d 1572, 1582, 221 U.S.P.Q. (BNA) 929, 93 (Fed. Cir. 1984) (this court will infer findings that were obviously necessary to the court's opinion).

Du Pont argues that, because its emulsion product was patented after the '978 patent issued, its product avoids infringement by equivalence. According to Du Pont, "*so long as direct infringement is lacking*, the grant of a patent to an accused infringer constitutes a prima facie determination of non-equivalence and, accordingly, of non-infringement" (Du Pont's emphasis). Atlas disagrees. [**32] So do we.

Du Pont concedes that, if Atlas patents A + B + C and Du Pont then patents the improvement A + B + C + D, Du Pont is liable to Atlas for any manufacture, use, or sale of A + B + C + D because the latter directly

infringes claims to A + B + C. Du Pont urges, however, that it is not liable for manufacture, use, or sale of patented improvement A + B + C', even though A + B + C' is "equivalent" to A + B + C. We reject Du Pont's attempted distinction. Whether Du Pont makes A + B + C + D or A + B + C', Du Pont has used the gist of Atlas' invention to devise a patentable composition. There is no compelling reason to hold Du Pont liable for infringement in one instance but not the other. [3]

> 3 Of course, if A + B + C' were patented because of unexpected results, those unexpected results might prompt a finding of no equivalence. That finding, however, would exist because, under the *Graver Tank* tripartite test, the "results" achieved by the claimed and accused products would be substantially different. The district court in this case did not find any such unexpected results. Though it found that Du Pont's products were more stable than those of the '978 patent, that is not necessarily inconsistent with equivalence. Equivalence does not require that the claimed invention and accused product have identical results; the results can be substantially the same and the accused product can be an improvement. *Perkin-Elmer Corp. v. Computervision Corp.*, 732 F.2d at 901-02, 221 U.S.P.Q. (BNA) at 679-80; *Decca Ltd. v. United States*, 210 Ct. Cl. 546, 544 F.2d 1070, 1080-81, 191 U.S.P.Q. (BNA) 439, 448 (1976).

[**33] We agree with *Bendix Corp. v. United States*, 199 U.S.P.Q. (BNA) 203 (Ct. Cl. Trial Div. 1978), *aff'd*, 220 Ct. Cl. 507, 600 F.2d 1364, 204 U.S.P.Q. (BNA) 617 (1979). There the trial judge said that [HN18]where defendant has appropriated the material features of the patent in suit, infringement will be found "even when those features have been supplemented and modified to such an extent that the defendant may be entitled to a patent for the improvement." 199 U.S.P.Q. (BNA) at 221-22. Though Du Pont argues that cases from other courts support a contrary result, we are not bound by those cases and in any event find them unpersuasive. [4]

> 4 We are bound by opinions of our predecessor courts, the Court of Claims and CCPA. *South Corp. v. United States*, 690 F.2d 1368, 215 U.S.P.Q. (BNA) 657 (Fed. Cir. 1982).

More persuasive is the reasoning of *Herman v. Youngstown Car Mfg. Co.*, 191 F. 579, 584-85 (6th Cir. 1911). After finding equivalence, the court rejected appellant's contention that its receipt of a [**34] patent negates infringement:

> [HN19]A patent is not the grant of a right to make or use or sell. It does not, directly or indirectly, imply any such right. It grants only the right to exclude others. The supposition that a right to make is created by the patent grant is obviously inconsistent with the established distinctions between generic and specific patents, and with the well-known fact that a very considerable portion of the patents [*1581] granted are in a field covered by a former relatively generic or basic patent, are tributary to such earlier patent, and cannot be practiced unless by license thereunder.
>
> Another reason sometimes advanced for supposing that the structure of the second does not infringe the claim of the first patent is that the Patent Office has declared that a patentable difference exists. The premise is sound, but not the conclusion. [HN20]In examining the second application, the Patent Office has no concern with the scope of the claim of the first, and does not and must not pay any attention thereto. It is concerned only with the early disclosure by the specification and drawings. [HN21]Patentable difference does not of itself tend to negative infringement. [**35] It may just as well be based upon infringement, plus improvement; and improvement may lie in addition, simplification, or variance.

See also Sanitary Refrigerator Co. v. Winters, 280 U.S. 30, 43, 74 L. Ed. 147, 50 S. Ct. 9 (1929) (where there is substantiality of function, way, and result, infringement cannot be avoided by any presumptive validity attaching to the issuance of a patent to the infringer); *Sure Plus Mfg. Co. v. Kobrin*, 719 F.2d 1114, 1117 (11th Cir. 1983) (no presumption of non-infringement arises from the issuance of a patent to the infringer); *Freeman v. Altvater*, 66 F.2d 506, 512 (8th Cir.), *cert. denied*, 290 U.S. 696, 78 L. Ed. 598, 54 S. Ct. 132 (1933) (the court after finding equivalence stated that the issuance of a patent merely raises a presumption of validity, not a presumption of non-infringement).

Du Pont contends that one skilled in the art in 1966 would not have known that the '978 and Du Pont products were equivalent. [HN22]It is not a requirement of equivalence, however, that those skilled in the art

know of the equivalence when the patent application is filed or the patent issues. That question is determined as of the time infringement [**36] takes place. In *Hughes Aircraft Co. v. United States*, 717 F.2d 1351, 1365, 219 U.S.P.Q. (BNA) 473, 483 (Fed. Cir. 1983), this court held that devices changing the patented invention with advances developed subsequent to the patent could infringe under the doctrine of equivalents. *See also American Hosp. Supply Corp. v. Travenol Labs., Inc.*, 745 F.2d 1, 9, 223 U.S.P.Q. (BNA) 577, 583 (Fed. Cir. 1984).

Du Pont also argues that Atlas is "estopped" from asserting that the '978 claims cover the use of an oil-in-water emulsifier such as sodium oleate because Atlas was unable to use that type of emulsifier effectively. We reject Du Pont's argument on two grounds.

First, [HN23]finding equivalence is not inconsistent with a patentee's unsuccessful attempt to make the accused product. The focus in assessing equivalence is on whether the accused product performs substantially the same as the claimed product in function, way and result -- it is not on the patentee's ability to devise a product equivalent to the patented product. Indeed, the patentee's incentive to devise an equivalent product is often less than a competitor's, which alone may account for the competitor's success and [**37] the patentee's failure in devising the equivalent product. *See, e.g., Leesona Corp. v. Varta Batteries, Inc.*, 522 F. Supp. 1304, 1328, 213 U.S.P.Q. (BNA) 222, 241 (S.D.N.Y. 1981).

Second, the record submitted to this court makes no reference to any type of estoppel. That strongly suggests that estoppel was not raised before the district court. *Bockoven v. Marsh*, 727 F.2d 1558, 1566 (Fed. Cir. 1984). Because [HN24]a party may generally not argue on appeal an issue not raised below, *Weinar v. Rollform Inc.*, 744 F.2d 797, 804, 223 U.S.P.Q. (BNA) 369, 372 (Fed. Cir. 1984); *Underwater Devices Inc. v. Morrison-Knudsen Co.*, 717 F.2d 1380, 1388, 219 U.S.P.Q. (BNA) 569, 575 (Fed. Cir. 1983), the estoppel argument is not properly before us.

Du Pont also argues that, because its product is formed in situ, it is different from the claimed product. It is the claimed product, however, not the process of forming it, that is involved. The district court found that the Du Pont emulsion, though it uses what is normally an oil-in-water emulsifier, "acts as a water-in-oil emulsifier", "caus[ing] a water-in-oil emulsion to form", [*1582] and is otherwise substantially the same [**38] as the '978 emulsion. Those findings have not been shown to be clearly erroneous.

Du Pont further contends that the district court erred in considering the "heart of the invention" in its infringement analysis. We disagree. [HN25]Although there is no legally recognized "essence" or "heart" of the invention in determining validity, *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 U.S.P.Q. (BNA) 303 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851, 105 S. Ct. 172, 83 L. Ed. 2d 107, 53 U.S.L.W. 3239 (1984), it can be applicable in a determination of infringement under the doctrine of equivalents. *Medtronic, Inc. v. Cardiac Pacemakers, Inc.*, 721 F.2d 1563, 1567, 220 U.S.P.Q. (BNA) 97, 101 (Fed. Cir. 1983). Moreover, the district court's "heart of the invention" analysis was supplemental to its finding that the *Graver Tank* tripartite test was satisfied.

Finally, Du Pont argues that the district court erred in not addressing in its opinion which of the individual claims are infringed. However, the district court specified the infringing claims in its judgment, and [HN26]we review judgments, not statements in opinions. *See, e.g., Lindemann Maschinenfabrik GMBH* [**39] *v. American Hoist & Derrick Co.*, 730 F.2d at 1463, 221 U.S.P.Q. (BNA) at 489. Reviewing the judgment, we conclude that the district court did not commit clear error in finding infringement of the claims on appeal.

VIII. *Conclusion*

Having considered all of Du Pont's arguments, the district court's decision that the '978 patent claims on appeal (1-5, 7, 12-14, and 16-17) are not invalid under 35 U.S.C. §§ 102, 103, and 112, that there was no inequitable conduct before the PTO, and that the claims on appeal were infringed, is *affirmed*.

AFFIRMED.

IN RE ALFRED MARZOCCHI AND RICHARD C. HORTON

No. 8431

United States Court of Customs and Patent Appeals

58 C.C.P.A. 1069; 439 F.2d 220; 1971 CCPA LEXIS 372; 169 U.S.P.Q. (BNA) 367

Oral argument January 7, 1971
April 15, 1971

PRIOR HISTORY: [***1] APPEAL from Patent Office, Serial No. 470,618

DISPOSITION: Modified.

CASE SUMMARY:

PROCEDURAL POSTURE: Appellants sought review of a decision of the Patent Office Board of Appeals, which affirmed the rejection of appellants' claims for obviousness and for inadequate disclosure, pursuant to 35 U.S.C.S. §§ 103, 112, respectively, such claims involving a technique for improving adhesion between glass fibers and vinyl resins.

OVERVIEW: Appellants asserted on appeal that improper conclusions were drawn from the Werner teaching to reject, based on obviousness pursuant to 35 U.S.C.S. § 103, claims that would improve the bonding relationship between glass and vinyl resins. The court disagreed, saying that in the absence of evidence disputing the Werner suggestion that the vinyl pyrrolidone moiety alone produces the desired adhesion enhancement, it must be accepted as fact. The inference that one of ordinary skill would recognize that a monomer possessed the same characteristic, was inescapable, the court explained. But obviousness claims had to be rejected. As to claims rejected for inadequate disclosure based on 35 U.S.C.S. § 112, the court reversed, finding insufficient justification for holding that appellants' disclosure concerning polyethyleneamine as an adhesion enhancer was not enabling. Despite being generic, the term had to be considered as including a number of compounds having the desired efficacy, absent evidence disputing that conclusion.

OUTCOME: The decision affirming the rejection of appellants' claims for obviousness was affirmed, because the technique for improving glass/vinyl-binding characteristics could be inferred from existing teaching, but rejection of the claims for inadequate disclosure was reversed, because the assertion of a generic term had to be considered to encompass numerous compounds having the desired efficacy, absent contrary evidence.

LexisNexis(R) Headnotes

Patent Law > Jurisdiction & Review > Standards of Review > General Overview
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
[HN1]In the absence of anything to rebut an assertion, which is reasonable on its face, a court is constrained to accept it as fact.

Patent Law > Claims & Specifications > Description Requirement > General Overview
Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
[HN2]A specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter

sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of 35 U.S.C.S. § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. Assuming that sufficient reason for such doubt does exist, a rejection for failure to teach how to make and/or use will be proper on that basis; such a rejection can be overcome by suitable proofs indicating that the teaching contained in the specification is truly enabling.

Patent Law > Claims & Specifications > Enablement Requirement > Scope
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
Patent Law > U.S. Patent & Trademark Office Proceedings > Examinations > General Overview
[HN3]It is incumbent upon the Patent Office to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement.

COUNSEL: *Herman Hersh* (McDougall, Hersh, Scott & Ladd), attorney of record, for appellant. *Staelin & Overman, George A. Degnan,* of counsel.

S. Wm. Cochran for the Commissioner of Patents. *Fred W. Sherling,* of counsel.

OPINION BY: BALDWIN

OPINION

[**221] [*1070] Before RICH, ALMOND, BALDWIN, LANE, Associate Judges, and DURFEE, Judge, sitting by designation

BALDWIN, Judge, delivered the opinion of the court.

This is an appeal from the decision of the Patent Office Board of Appeals which affirmed the final rejection of claims 5 and 11 of appellants' application [1] under 35 USC 103 as unpatentable in view of Werner [2] and of claims 6 and 12 under 35 USC 112 as being based on an inadequate disclosure. Claims 4 and 10 stand allowed.

1 Serial No. 470,618, filed July 8, 1965, for "Fiber Coatings - Nitrogen Compounds for Improving Adhesion of Vinyl Polymers to Glass" as a continuation-in-part of Serial No. 96-106, filed March 16, 1961.

2 U.S. Patent No. 2,853,465, issued September 23, 1958.

The Invention

The subject matter of the claims on appeal involves a technique for improving the [***2] adhesion characteristics between glass fibers and vinyl polymer resins. Claim 5 is representative and reads as follows:

5. In the combination of glass fibers and a vinyl polymer resin composition present as a coating on the glass fiber surfaces, the improvement which comprises mixing the vinyl polymer resin, prior to coating of the glass fibers, with an amine compound in an amount corresponding to 2-10% by weight of the vinyl polymer resin, and in which the amine compound is monomeric vinyl pyrrolidone.

Claim 11 is drawn to the same concept as claim 5, but defines the invention as "a method of producing glass fibers coated with polyvinyl resin strongly bonded to the glass fiber surfaces." Claims 6 and 12 differ from claims 5 and 11 respectively solely in the recitation of "polyethyleneamine" as the critical "amine compound" additive.

The Section 103 Rejection

Claims 5 and 11 were rejected "as obvious in the sense of 35 USC 103 over Werner." Werner, the sole reference relied upon here, is addressed to the improvement in the bonding relationship between glass and polyvinyl halide resins. The pertinent disclosure is as follows [emphasis added]:

I have found that polyvinyl [***3] halide resins may be successfully modified so as to obtain excellent glass adhesion by employing a mixture of a polyvinyl halide and a polymer of N-vinyl pyrrolidone. By employing a mixture containing from 80 to 97% of a polyvinyl halide and from 20 to 3% of a polymer of N-vinyl pyrrolidone, which term includes homopolymers of vinyl pyrrolidone and copolymers with other polymerizable monomers, a composition is obtained having extremely high adhesion to all glass surfaces.

[*1071] On the basis of this teaching the examiner took the position, accepted by the [**222] board, that the claimed use of monomeric vinyl pyrrolidone rather than Werner's polymeric vinyl pyrrolidone would be obvious to one of ordinary skill in the art since Werner's teaching would indicate to "one skilled in the art * * * that it is the vinyl pyrrolidone moiety that is enhancing the adhesion." It was also suggested by the examiner that since the claims recite no temperature conditions for the coating operation and since monomers polymerize when heated, the claims could possibly cover circumstances wherein the monomer is polymerized during application. The board appears to have accepted this suggestion [***4] and to have extended it even further. It stated:

All of Werner's examples specify heating at elevated temperatures (110 degrees C.-130 degrees C., 165 degrees C., 325 degrees F., 350 degrees F.) with and without elevated pressures. Appellants' specification says nothing about retaining the vinyl pyrrolidone in monomeric form, much less anything about "maximizing adhesion" by preventing polymerization. Indeed, the very designation of the vinyl pyrrolidone as a "monomeric" material introduced into a polymer system for the purpose of altering the properties of such system implies subsequent polymerization of the monomer. Appellants' further argument that the monomer has entirely different capabilities and solubilities than the polymer is also unpersuasive.

Appellants' position on appeal in response to these assertions by the examiner and board is largely to stress again the "marked difference between the properties and characteristics of a polymer as compared to a monomer," and to object to the "purely conjectural" assertion that the monomer polymerizes in the coating after it is applied. Additionally, appellants make the following contention:

Even if it were assumed that [***5] appellants' monomeric vinyl pyrrolidone is polymerized when present in the polyvinyl chloride coating, there is no teaching or suggestion in Werner that the use of monomeric vinyl pyrrolidone has any efficacy whatsoever in compositions of the type disclosed and claimed. The basis suggested by the Patent Office for the rejection is tantamount to the allegation it would be "obvious to try" the monomer. This "test" of obviousness has been frequently repudiated by this court.

The sole issue is, of course, whether the Werner teaching does suggest to a person having ordinary skill in this art that the use of monomeric vinyl pyrrolidone would have the efficacy indicated in the appealed claims. We agree with appellants that whether the monomer polymerizes is irrelevant, at least in this regard. What is relevant, however, and here determinative, is the examiner's assertion that the Werner teaching would suggest that it is the vinyl pyrrolidone moiety alone and not some other characteristic peculiar to a polymer which is efficacious in producing the desired adhesion enhancement. [3] [*1072] [HN1]In the absence of anything to rebut this assertion, which is reasonable on its face, we are [***6] constrained to accept it as fact. The inferences which follow from such fact, i.e., that the monomer would possess this same characteristic and that one of ordinary skill would recognize such fact, are inescapable.

> 3 Indeed, the reasonableness of such an assertion is confirmed by the very disclosure contained in appellants' application which indicates that efficacious adhesion enhancers are those "organic nitrogenous compounds which are characterized both by an organic constitution which is compatible with the vinyl polymers and by a polarity expressed in the nitrogen function." As also pointed out by appellants in their brief (about which more will be said later), the nature of the present invention resides in the use of amine compounds, broadly, as adhesion enhancers.

It is acknowledged that the above line of reasoning may be viewed as being tantamount to drawing the inference that, to one possessing the ordinary level of skill in this art, it would be "obvious [**223] to try" the monomer. Nevertheless, such an inference of fact may, at times, be enough to justify drawing the ultimate conclusion of law that the claimed subject matter as a whole would have been obvious [***7] under section 103. We are satisfied that the circumstances of this case justify an initial conclusion of obviousness. Since the record before us contains nothing to rebut that conclusion, the decision with regard to claims 5 and 11 must be affirmed.

The Section 112 Rejection

Claims 6 and 12, which recite the use of "polyethyleneamine" as the adhesion enhancer, were criticized by the examiner as being based on a disclosure which was not enabling under the first paragraph of 35 USC 112. The board affirmed his rejection of those claims with the following comment.

The term is obviously generic to a considerable number of compounds varying in the number of ethylene groups, the number of amine groups and the relationship of the polyethylene groups to the amine groups, and accordingly does not provide a reasonable guide for those seeking to improve the adherence of vinyl resins to glass.

[1] We will reverse the board's decision on this rejection since we are unable to find sufficient justification for the holding that appellants' disclosure is not enabling.

Turning specifically to the objections noted by the board as indicated above, it appears that these comments indicate [***8] nothing more than a concern over the breadth of the disputed term. If we are correct, then the relevance of this concern escapes us. It has never been contended that appellants, when they included the disputed term in their specification, intended only to indicate a single compound. Accepting, therefore, that the term is a generic one, its recitation must be taken as an assertion by appellants that all of the "considerable [*1073] number of compounds" which are included within the generic term would, as a class, be operative to

produce the asserted enhancement of adhesion characteristics. The only relevant concern of the Patent Office under these circumstances should be over the truth of any such assertion. The first paragraph of § 112 requires nothing more than objective enablement. How such a teaching is set forth, either by the use of illustrative examples or by broad terminology, is of no importance.

[2] As a matter of Patent Office practice, then, [HN2]a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to [***9] be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. Assuming that sufficient reason for such doubt does exist, a rejection for failure to teach how to make and/or use will be proper on that basis; such a rejection can be overcome by suitable proofs indicating that the teaching contained in the specification is truly enabling.

[3] In the field of chemistry generally, there may be times when the well-known unpredictability of chemical reactions will alone be enough to create a reasonable doubt as to the accuracy of a particular broad statement put forward as enabling support for a claim. This will especially be the case where the statement is, on its face, contrary to generally accepted scientific principles. Most often, additional factors, such as the teachings in pertinent references, [4] will be available to substantiate any doubts that the asserted scope of objective enablement [**224] is in fact commensurate with the scope of protection sought and to support any demands based thereon [***10] for proof. In any event, [HN3]it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure. Cf. In re Gazave, 54 CCPA 1524, 379 F.2d 973, 154 USPQ 92 (1967); In re Chilowsky, 43 CCPA 775, 229 F.2d 457, 108 USPQ 321 (1956).

4 Not necessarily prior art references, it should be noted, since the question would be regarding the accuracy of a statement in the specification, not whether that statement had been made before.

In the present case, the circumstances we see do not support the reasonableness of any doubts which the Patent Office might have had [*1074] concerning the adequacy of appellants' specification disclosure to support these claims. In fact, those circumstances tend to strengthen rather than weaken appellants' claim to the breadth of protection they seek. In the first place, it has not been [***11] asserted by the Patent Office that the chemical properties of known polyethyleneamines vary to such an extent that it would not be expected by one of ordinary skill in this art that any such compound would possess the necessary capability of enhancing adhesion. Additionally, we note that polyethyleneamine is listed in appellants' specification as being only one of a much larger class of amine compounds possessing this necessary characteristic. Finally, we recognize (as did the examiner) the generic nature of appellants' broader concept, i.e., that the desired property of adhesion enhancement stems largely from the amine moiety. It does appear that variation of certain of the secondary factors mentioned by the examiner, such as molecular weight or proportion of ethylene groups, might influence to some degree or even mask the essential "amine" property of the polyethylene amine or its obviously equally essential compatibility with vinyl polymers. However, we see no basis to conclude that the ready avoidance of this result would not be within the level of ordinary skill in this art. Compare In re Skrivan, 57 CCPA 1201, 427 F.2d 801, 166 USPQ 85 (1970).

Taking all these circumstances [***12] into consideration, we are constrained to conclude that the record before us contains insufficient grounds for questioning the accuracy of appellants' teaching that any polyethyleneamine (obviously excepting those whose essential "amine" characteristics and compatibility with vinyl polymers would be masked by the secondary factors mentioned) will function to accomplish the asserted result. It follows that claims 6 and 12 must be held to be supported by a disclosure which is in compliance with the requirements of the first paragraph of 35 USC 112.

Summary

The decision of the board regarding claims 5 and 11 is affirmed; that dealing with claims 6 and 12 is reversed.

IN RE STEPHEN E. WRIGHT

92-1437

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

999 F.2d 1557; 1993 U.S. App. LEXIS 18347; 27 U.S.P.Q.2D (BNA) 1510

July 20, 1993, Decided

PRIOR HISTORY: [**1] Appealed from: U.S. Patent and Trademark Office Board of Patent Appeals and Interferences. Serial No. 06/914,620

DISPOSITION: AFFIRMED

CASE SUMMARY:

PROCEDURAL POSTURE: Appellant sought review of an order of the United States Patent and Trademark Office Board of Patent Appeals and Interferences sustaining the rejection of his claims directed to processes for producing vaccines against certain viruses as unsupported by an enabling disclosure.

OVERVIEW: Appellant filed claims to patent processes for producing vaccines against certain viruses, to patent vaccines produced by the processes, and to patent methods of using the vaccines. However, although appellant's specification provided a general description of the processes, vaccines and methods of use, he only provided one working example. Appellee Board thus rejected appellant's claims, since they were not supported by an enabling disclosure and since one of ordinary skill in the art would have to engage in undue experimentation to practice the subject matter of the claims given the breadth of the claims, the unpredictability in the art and the limited guidance provided by appellant in his application. The court affirmed, since appellee set forth a reasonable explanation as to why the claim was not adequately enabled by the description of the invention in the specification and appellant failed to provide suitable proofs indicating that the specification was enabling.

OUTCOME: The order sustaining rejection of appellant's claims was affirmed because appellant failed to provide proofs indicating his specification was enabling.

LexisNexis(R) Headnotes

Civil Procedure > Appeals > Standards of Review > De Novo Review
Patent Law > Claims & Specifications > Definiteness > General Overview
Patent Law > U.S. Patent & Trademark Office Proceedings > General Overview
[HN1]The first paragraph of 35 U.S.C.S. § 112 requires that the specification of a patent contain a written description of the claimed invention and the manner and process of making and using that invention in such full, clear, concise, and exact terms as to enable any person skilled in the art to which that invention pertains, or with which it is most nearly connected, to make and use that invention. As a statutory requirement, enablement is a question of law that the court reviews de novo; however, the court reviews for clear error any underlying facts found by the Board of Patent Appeals and Interferences in rendering its enablement determination.

Patent Law > Claims & Specifications > Description Requirement > General Overview
Patent Law > Claims & Specifications > Enablement Requirement > Standards & Tests
Patent Law > Inequitable Conduct > General Overview

[HN2]To be enabling, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without "undue experimentation." The first paragraph of 35 U.S.C.S. § 112 requires that the scope of protection sought in a claim bear a reasonable correlation to the scope of enablement provided by the specification. Nothing more than objective enablement is required, and therefore it is irrelevant whether this teaching is provided through broad terminology or illustrative examples.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Inequitable Conduct > General Overview
[HN3]When rejecting a claim under the enablement requirement of 35 U.S.C.S. § 112, the United States Patent and Trademark Office (PTO) bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application; this includes, of course, providing sufficient reasons for doubting any assertions in the specification as to the scope of enablement. If the PTO meets this burden, the burden then shifts to the applicant to provide suitable proofs indicating that the specification is indeed enabling.

COUNSEL: Peter M. Peer, Mallinckrodt & Mallinckrodt, of Salt Lake City, Utah, argued for appellants. With him on the brief was Philip A. Mallinckrodt.

Teddy S. Gron, Associate Solicitor, Office of the Solicitor, of Arlington, Virginia, argued for appellee. With him on the brief was Fred E. McKelvey, Solicitor. Of counsel were Richard E. Schafer, John W. Dewhirst, Albin F. Drost and Lee E. Barrett.

JUDGES: Before RICH, NEWMAN, and RADER, Circuit Judges.

OPINION BY: RICH

OPINION

[*1558] RICH, *Circuit Judge.*

Dr. Stephen E. Wright appeals from the January 16, 1992 decision of the Board of Patent Appeals and Interferences (Board) of the United States Patent and Trademark Office (PTO) sustaining the Examiner's rejection of claims 1-23, 15-42, and 45-48 of application Serial No. 06/914,620 [1] under 35 [*1559] U.S.C. § 112, first paragraph, as unsupported by an enabling disclosure. [2] We affirm.

1 Application Serial No. 06/914,620, filed on October 2, 1986, is a continuation of Serial No. 06/469,985, filed February 25, 1983, now abandoned.

[**2]

2 In an April 12, 1992 reconsideration decision, the Board denied Wright's request that it modify its original decision.

I. BACKGROUND

A. *The Invention*

The claims on appeal are directed to processes for producing live, non-pathogenic vaccines against pathogenic RNA viruses (claims 1-10, 22-37, 40, 45, and 46), vaccines produced by these processes (claims 11, 12, 15-21, 38, and 39), and methods of using certain of these claimed vaccines to protect living organisms against RNA viruses (claims 41 and 42). Wright's specification provides a general description of these processes, vaccines, and methods of use, but only a single working example.

In this example, Wright describes the production of a recombinant vaccine which confers immunity in chickens against the RNA tumor virus known as Prague Avian Sarcoma Virus (PrASV), a member of the Rous Associated Virus (RAV) family. To produce this vaccine, Wright first identified the antigenic gene region of the genome of PrASV as being in the envelope A *(env* A) gene region of this virus, and then isolated and cloned a large quantity of this antigenic [**3] gene region. Following cloning, Wright introduced by transfection the cloned *env* A genes into C/O cells, a particular chicken embryo cell line. The C/O cells were then infected with the endogenous, non-oncogenic, O-type Rous Associated Virus (RAV-O) and incubated. Genetic recombination and viral replication occurred during incubation, resulting in an impure vaccine containing particles of the recombinant virus referred to as RAV-O Ac<n>, or RAV-O-A. [3] Wright then purified this vaccine to obtain a vaccine containing only genetic recombinant RAV-Ac<n> virus particles. The Examiner ultimately allowed claims 13, 14, 43, and 44, which are specific to the particular process and vaccine disclosed in this example. [4]

3 Transfection, infection, genetic recombination, and viral replication collectively constitute a procedure known as marker rescue.

4 The allowed claims read:

> *Claim 14* A vaccine according to claim 13, wherein the vaccine has been purified by selection for the expression of the antigenic genome.
>
> *Claim 43* A process for producing a live, non-pathogenic, recombinant vaccine conferring immunity against the PrASV avian tumor virus in chickens, comprising inserting the PrASV env A gene into a RAV-O virus by marker rescue such that said PrASV env A gene replaces the endogenous envelope gene of the RAV-O virus; and selecting for the recombinant in C/E cells.
>
> *Claim 44* A live, non-pathogenic, recombinant vaccine conferring immunity against the PrASV avian tumor virus in chickens, in which vaccine the PrASV env A gene has been inserted into a RAV-O virus by marker rescue to replace the endogenous envelope gene of the RAV-O virus, and the recombinant has been selected for in C/E cells.

[**4] Wright seeks allowance, however, of claims which would provide, in varying degrees, a much broader scope of protection than the allowed claims. For example, independent process claim 1 reads:

> A process for producing a live non-pathogenic vaccine for a pathogenic RNA virus, comprising the steps of identifying the antigenic and pathogenic gene regions of said virus; performing gene alteration to produce a genome which codes for the antigenicity of the virus, but does not have its pathogenicity; and obtaining an expression of the gene.

Dependent claims 2-10, 22-35, and 40 recite additional limitations to this process. Independent claims 36 and 45 and claims 37 and 46 dependent there from, respectively, are also directed to processes for producing vaccines.

Independent product claim 11 reads:

> A live, non-pathogenic vaccine for a pathogenic RNA virus, comprising an immunologically effective amount of a viral antigenic, genomic expression having an antigenic [*1560] determinant region of the RNA virus, but no pathogenic properties.

Dependent claims 15-21 recite additional limitations to this vaccine. Independent claims 38 and 47 and claims 39 and 48 dependent therefrom, respectively, [**5] are also directed to vaccines.

Dependent claims 41 and 42 recite methods of protecting living organisms against RNA viruses, which comprise introducing into a host an immunologically effective amount of the vaccine of claims 11 and 38, respectively.

B. *The Rejection*

The Examiner took the position in her Examiner's Answer that the claims presently on appeal are not supported by an enabling disclosure because one of ordinary skill in the art would have had to engage in undue experimentation in February of 1983 (the effective filing date of Wright's application) to practice the subject matter of these claims, given their breadth, the unpredictability in the art, and the limited guidance Wright provides in his application. The Examiner noted that many of Wright's claims read on vaccines against *all* pathogenic RNA viruses, even though RNA viruses are a very diverse and genetically complex group of viruses which include, among others, acquired immunodeficiency syndrome (AIDS) viruses, leukemia viruses, and sarcoma viruses. The Examiner argued that Wright's single working example merely evidenced that Wright had obtained successfully a particular recombinant virus vaccine, and [**6] that this single success did not provide "sufficient likelihood" that other recombinant RNA viruses could be constructed without undue experimentation, or if they were constructed, that they would be useful in the design of live viral vaccines. The Examiner noted the inability of the scientific community to develop an efficacious AIDS virus vaccine for humans despite devoting a considerable amount of time and money to do so.

The Examiner further argued that, even though retroviruses as a class may exhibit similar gene order and possess envelope proteins, this alone does not support a general conclusion that all RNA virus envelope proteins will confer protection against the corresponding virus. The Examiner asserted that this held true even among avian RNA tumor viruses. At page 11 of her Answer, the Examiner stated that "one envelope gene's immunogenicity cannot be extrapolated to another envelope gene. The efficacy of each should be ascertained individually."

To support the foregoing, the Examiner relied upon an article by Thomas J. Matthews et al., *Prospects for Development of a Vaccine Against HIV,* in Human Retroviruses, Cancer, and AIDS: Approaches to Prevention and Therapy [**7] 313-25 (1988). This article indicates that AIDS retroviruses, which represent only a subset of all RNA viruses, were known even as late as 1988 to show great genetic diversity, including

divergent virus envelopes. It further indicates that, although AIDS retroviruses elicited strong immune responses in goats and chimps in 1988, the resulting antibodies did not prevent retrovirus infectivity. Moreover, this article also recognizes at page 321 that, as of 1988, animal models for HIV infection and disease were likely to be imperfect, and therefore testing of primary vaccine candidates in man was necessary to determine safety, immunogenicity and efficacy.

Finally, the Examiner also argued that, irrespective of immunogenicity and vaccine considerations, the methods of identification, isolation, cloning, and recombination which Wright describes in his application in only a very general manner were not so developed in 1983 as to enable, without undue experimentation, the design and production of recombinant virus vaccines against any and all RNA viruses. The Examiner also asserted that the considerable amount of time and effort that it took Wright to construct the particular avian recombinant [**8] virus described in his single working example and to establish its efficacy as a vaccinating agent illustrates the amount of undue experimentation that would have been required in February of 1983 to practice Wright's invention, especially given that the efficacy of the developed virus could not be extrapolated with any certainty to other recombinant viruses at that time.

[*1561] C. *The Board Decision*

In its January 16, 1992 decision, [5] the Board held that the Examiner did not err in questioning the enablement of the physiological activity required by the appealed claims, given the breadth of these claims and the fact that a vaccine must by definition provoke an immunoprotective response upon administration. The Board found that Wright had failed to establish that the general description of his invention set forth in his application was anything more, in February of 1983, than an invitation to experiment. The Board agreed with the Examiner that this general description does not set forth such sufficient detailed guidance that one of ordinary skill in the art would have had any reasonable expectation of success in constructing other vaccines against other RNA viruses. The Board additionally [**9] noted that the Examiner only allowed the claims limited to Wright's single working example after Wright submitted *in vivo* evidence of the efficacy of this vaccine.

5 The Board stated in its opinion that it was affirming the Examiner's rejection under section 112 for the reasons set forth in the Examiner's Answer and that the Board's added comments were for emphasis only.

The Board further held that the record did not support Wright's arguments that his single working example enables some of his dependent claims which are closer in scope to the allowed claims than to independent claims 1 and 11. The Board found that, even if Wright was correct in stating that it was generally known that an "immune response" is assured by use of an antigenic envelope protein, the record did not establish that such an "immune response" would have been an immunoprotective one, or moreover, that one skilled in this art would have expected such a result in February of 1983. The Board relied upon the Matthews et al. article as evidencing [**10] that "the mere use of an envelope protein gene in the present invention is not seen to necessarily result in the obtention of successful vaccines throughout the scope of even these more limited claims." Bd. Dec. at 6.

As to Wright's request that the Board consider several declarations and exhibits of record, the Board stated that it found no reason to consider this evidence with any particularity since Wright had failed to advance any specific arguments based on this evidence or to explain its relevance.

II. DISCUSSION

A.

[HN1]The first paragraph of 35 USC § 112 requires that the specification of a patent contain a written description of the claimed invention and the manner and process of making and using that invention in such full, clear, concise, and exact terms as to enable any person skilled in the art to which that invention pertains, or with which it is most nearly connected, to make and use that invention. As a statutory requirement, enablement is a question of law that we review de novo; however, we review for clear error any underlying facts found by the Board in rendering its enablement determination. *In re Vaeck,* 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991); [**11] *In re Wands,* 858 F.2d 731, 735, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).

Although not explicitly stated in section 112, [HN2]to be enabling, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without "undue experimentation." *Vaeck,* 947 F.2d at 495, 20 USPQ2d at 1444; *Wands,* 858 F.2d at 736-37, 8 USPQ2d at 1404; *In re Fisher,* 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970) (the first paragraph of section 112 requires that the scope of protection sought in a claim bear a reasonable correlation to the scope of enablement provided by the specification). Nothing more than objective enablement is required, and therefore it is irrelevant whether this teaching is provided through broad terminology or

illustrative examples. *In re Marzocchi,* 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971).

[HN3]When rejecting a claim under the enablement requirement of section 112, the PTO bears an initial burden of setting [**12] forth a reasonable explanation as to why it believes [*1562] that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application; this includes, of course, providing sufficient reasons for doubting any assertions in the specification as to the scope of enablement. If the PTO meets this burden, the burden then shifts to the applicant to provide suitable proofs indicating that the specification is indeed enabling. *Marzocchi,* 439 F.2d at 223-24, 169 USPQ at 369-70.

B.

In the present case, the PTO set forth a reasonable basis for finding that the scope of the appealed claims is not enabled by the general description and the single working example in the specification. Consequently, the burden shifted to Wright to present persuasive arguments, supported by suitable proofs where necessary, that the appealed claims are truly enabled. Wright failed to meet this burden.

Both the Examiner and the Board correctly pointed out that Wright's appealed claims are directed to vaccines, and methods of making and using these vaccines, which must by definition trigger [**13] an immunoprotective response in the host vaccinated; mere antigenic response is not enough. [6] Both also correctly pointed out that Wright attempts to claim in many of the appealed claims *any and all* live, non-pathogenic vaccines, and processes for making such vaccines, which elicit immunoprotective activity in *any* animal toward *any* RNA virus. In addition, both properly stressed that many of the appealed claims encompass vaccines against AIDS viruses and that, because of the high degree of genetic, antigenic variations in such viruses, no one has yet, years after his invention, developed a generally successful AIDS virus vaccine.

6 Wright defines "vaccine" at page 1 of his specification as being a "material which induces an organism to acquire immunity against disease." Furthermore, as noted by the Board, the *Dictionary of Biochemistry* 330 (John Wiley & Sons 1975), defines "vaccine" as a suspension of antigens derived from viruses or bacteria that, upon administration, will produce active immunity and provide protection against those viruses or bacteria or related viruses or bacteria.

[**14] The Matthews et al. article, published approximately 5 years after the effective filing date of Wright's application, adequately supports the Examiner's and the Board's position that, in February of 1983, the physiological activity of RNA viruses was sufficiently unpredictable that Wright's success in developing his specific avian recombinant virus vaccine would not have led one of ordinary skill in the art to believe reasonably that all living organisms could be immunized against infection by any pathogenic RNA virus by inoculating them with a live virus containing the antigenic code but not the pathogenic code of that RNA virus. The general description and the single example in Wright's specification, directed to a uniquely tailored *in vitro* method of producing in chicken C/O cells a vaccine against the PrASV avian tumor virus containing live RAV-Ac<n> virus particles, did nothing more in February of 1983 than invite experimentation to determine whether other vaccines having *in vivo* immunoprotective activity could be constructed for other RNA viruses.

Wright argues that he has constructed successfully an *env* C recombinant vaccine according to the present invention [**15] and that certain recombinant AIDS virus vaccines carrying SIV (simian immunodeficiency virus) and HIV (human immunodeficiency virus) envelope genes have been produced which confer protective immunity in the animal models where they have been tested, [7] and that these developments illustrate that the art is not so unpredictable as to require undue experimentation. However, all of these developments occurred after the effective [*1563] filing date of Wright's application and are of no significance regarding what one skilled in the art believed as of that date. [8] Furthermore, the fact that a few vaccines have been developed since the filing of Wright's application certainly does not by itself rebut the PTO's assertions regarding undue experimentation. Moreover, whether a few AIDS virus vaccines have been developed which confer immunity in some animal models is not the issue. The Examiner made reference to the difficulty that the scientific community is having in developing generally successful AIDS virus vaccines merely to illustrate that the art is not even today as predictable as Wright has suggested that it was back in 1983.

7 *See* Shio-Luk Hu et al., *Protection of Macaques Against SIV Infection by Subunit Vaccines of SIV Envelope Glycoprotein gp160,* 255 Science 456-59 (1992); Shio-Luk Hu et al., *Neutralizing Antibodies Against HIV-1 BRU and SF2 Isolates Generated in Mice Immunized with Recombinant Vaccinia Virus Expressing HIV-1 (BRU) Envelope Glycoproteins and Boosted with Homologous gp 160,* 7 AIDS RESEARCH AND HUMAN RETROVIRUSES 616-20 (1991); Phillip W. Berman et al., *Protection of*

Chimpanzees from Infection by HIV-1 after Vaccination with Recombinant Glycoprotein gp120 but not gp160, 345 Nature 622-625 (1990).

[**16]

8 In his appeal to this court, Wright all too frequently slips into using the present tense to discuss the state of the art and what a skilled artisan would believe given Wright's success with one avian virus. We note, however, that the issue is not what the state of the art is today or what a skilled artisan today would believe, but rather what the state of the art was in February of 1983 and what a skilled artisan would have believed at that time. *Hybritech Inc. v. Monoclonal Antibodies, Inc.,* 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir.), *cert denied,* 480 U.S. 947 (1987); *In re Hogan,* 559 F.2d 595, 604, 194 USPQ 527, 535 (CCPA 1977). Wright's tendency to employ the present tense often makes it difficult to determine whether Wright is asserting that certain information was known prior to February of 1983 or simply that that information is now known in the art.

Wright also argues that several affidavits of record, namely, an October 22, 1984 declaration by Wright, an [**17] October 23, 1984 affidavit by O'Neill, and October 28, 1988 affidavits by Bennett and Burnett, successfully rebut the Board's and the Examiner's assertions regarding undue experimentation. However, Wright did not set forth in his brief to the Board any specific arguments regarding these affidavits, as required by 37 CFR 1.192(a), and therefore we are not required to address the arguments that Wright presents in this appeal regarding these affidavits. *Chester v. Miller,* 906 F.2d 1574, 1578 n.6, 15 USPQ2d 1333, 1337 n.6 (Fed. Cir. 1990); *In re Wiseman,* 596 F.2d 1019, 1022, 201 USPQ 658, 661 (CCPA 1979).

Nevertheless, we note that each of these affidavits fails in its purpose because each merely contains unsupported conclusory statements as to the ultimate legal question. [9] *See In re Buchner,* 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *In re Brandstadter,* 484 F.2d 1395, 1405-06, 179 USPQ 286, 293-94 (CCPA 1973). Furthermore, Burnett and Bennett do not even indicate in their affidavits that [**18] they actually reviewed the specification of Wright's application. In addition, although Wright states in his declaration that the individual steps making up his claimed process were "well within the skill of the art" at the time that he filed his application and makes reference to a list of publications that he contends supports this conclusory statement, a list which Wright also makes reference to in his arguments to this court, Wright fails to point out with any particularity in this declaration, or in his arguments to this court, how the listed documents evidence that a skilled artisan in February of 1983 would have been able to carry out, without undue experimentation, the identification, isolation, cloning, recombination, and efficacy testing steps required to practice the full scope of the appealed claims.

9 For example, O'Neill stated in his affidavit that the specification provided him "with sufficient information to produce a vaccine for any known pathogen in accordance with the procedural steps claimed" and that he could not "foresee any problem in producing similar vaccines for other pathogens." Bennett and Burnett merely declared in their affidavits that, at the time they worked with Wright, they believed that the specific vaccine that they were developing was simply one example within a broader concept. In his October 22, 1984 declaration, Wright stated that he knew "of no reason why the specific vaccine tested is not truly indicative of successful application of other embodiments of the invention within the purview of the teachings and claims of my patent application." Wright further stated that "[although the project has been primarily directed toward the production and testing of a specific vaccine for a specific virus, the concept has been inherently generic as claimed."

[**19] C.

Wright further argues that, even if those claims which provide a broad scope of protection are not enabled, this is not the case as to those claims restricted to vaccines against [*1564] avian tumor viruses. Wright maintains that there is no doubt that it was known in 1983 that the technique of producing a live vaccine proven effective for one particular strain of avian RNA viruses would be effective as to other strains of avian RNA viruses. Wright argues that the scientific literature supports the position that the art was predictable at least with respect to avian RNA viruses, because gene function and order are similar among all avian RNA viruses.

We are not persuaded. Wright has failed to establish by evidence or arguments that, in February of 1983, a skilled scientist would have believed reasonably that Wright's success with a particular strain of an avian RNA virus could be extrapolated with a reasonable expectation of success to other avian RNA viruses. Indeed, Wright has failed to point out with any particularity the scientific literature existing in February of 1983 that supports his position. Furthermore, Wright's May 17, 1989 declaration indicates that Wright himself believed [**20]

during the relevant time period that *in vivo* testing was necessary to determine the efficacy of vaccines. In this declaration, Wright stated in pertinent part:

> Preparation of a patent application following conception of the invention awaited such time as there was a reasonable expectation that the results of innoculation [sic] would be successful. *This became apparent only by reason of survival of the chickens that had been innoculated* [sic].

Wright now argues that all he meant by the foregoing was that *in vivo* efficacy testing was necessary for the first avian RNA viruses vaccine that he developed in order to prove his hypothesis. Wright asserts that, once his hypothesis had been proven, a skilled artisan would have expected that similar *in vivo* results could be obtained for vaccines developed for other avian retroviruses. However, a paper that Wright co-authored with David Bennett, titled "Avian Retroviral Recombinant Expressing Foreign Envelope Delays Tumor Formation of ASV-A-Induced Sarcoma," which was attached to a declaration by Wright dated November 19, 1985, suggests that, even as late as 1985, the genetic diversity existing among chickens alone required [**21] efficacy testing even among the members of this narrow group. [10] Accordingly, we see no error in the Board's finding that one skilled in the art would not have believed as early as February of 1983 that the success of Wright's one example could be extrapolated with a reasonable expectation of success to all avian RNA viruses.

> 10 The paper states in pertinent part: "Recent observations suggest line SC chickens do not respond well immunologically . . . Follow up experiments will include other chicken lines. Mechanisms of protection are also under study."

D.

Finally, Wright argues that each of the appealed claims should be considered independently to determine whether it satisfies the enablement requirement of section 112. This we have done. Wright's arguments to this court, however, are directed almost entirely to why he believes that the specification of his application enables the appealed claims as a whole or at least those claims limited to avian RNA viruses. Although Wright does refer in passing to each of the [**22] appealed claims, he does little more than recite the particular limitations recited in these claims, failing to point out how the enablement requirement is satisfied as to each of these claims independently. Consequently, Wright has failed to provide us with any justification for finding that the Board erred in sustaining the Examiner's rejection, even with respect to some of the more limited claims.

CONCLUSION

For the foregoing reasons, the decision of the Board is affirmed.

AFFIRMED

FALKO-GUNTER FALKNER, GEORG HOLZER, and FRIEDRICH DORNER, Appellants, v. STEPHEN C. INGLIS, MICHAEL E.G. BOURSNELL, and ANTHONY C. MINSON, Appellees.

05-1324, (Interference No. 105,187)

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

448 F.3d 1357; 2006 U.S. App. LEXIS 13127; 79 U.S.P.Q.2D (BNA) 1001

May 26, 2006, Decided

SUBSEQUENT HISTORY: Rehearing denied by, Rehearing, en banc, denied by Falkner v. Inglis, 2006 U.S. App. LEXIS 22630 (Fed. Cir., Aug. 24, 2006)
US Supreme Court certiorari denied by Falkner v. Inglis, 2007 U.S. LEXIS 1185 (U.S., Jan. 22, 2007)

PRIOR HISTORY: [**1] Appealed from: United States Patent and Trademark Office, Board of Patent Appeals and Interferences.

DISPOSITION: AFFIRMED.

CASE SUMMARY:

PROCEDURAL POSTURE: Appellant patent holder sued appellee patent applicant, alleging, inter alia, interference of a vaccine patent. The United States Board of Patent Appeals and Interferences held that the patent holder could not antedate the applicant's priority date and entered judgment against the patent holder. The patent holder appealed.

OVERVIEW: The only issue that needed to be resolved was whether the applicant's benefit applications adequately described and enabled a poxvirus-based vaccine. The application provided a detailed example of an embodiment that comprised not a poxvirus, but a herpesvirus, including the identity of the deleted essential sequences therein. Nevertheless, the Board did not err in finding that the applicant's claims were enabled as a matter of law given the extensive disclosure of the selection of an essential gene, its deletion or inactivation, and the production of a mutated virus with said deleted or inactivated gene, albeit for herpesvirus. The differences between the herpesviruses and poxviruses were well known and would have aided a person of ordinary skill in the art on applying the lessons of the herpesvirus example in the construction of poxvirus vaccines. Although the Board erred in its articulation of the written description standard, the error was harmless because the undisputed evidence supported the Board's ultimate conclusion that the accessible literature sources adequately described the invention.

OUTCOME: The judgment was affirmed.

LexisNexis(R) Headnotes

Patent Law > U.S. Patent & Trademark Office Proceedings > Interferences > Patentability & Priority Determinations

[HN1]Priority in an interference goes to the first to invent, but a rebuttable presumption exists that the inventors made their inventions in the chronological order of their effective filing dates, namely that the senior party invented first, 37 C.F.R. § 1.657(a) (2004), and the junior party bears the burden of proving otherwise, 37 C.F.R. § 1.657(b), such as by proving that she actually reduced the invention to practice before the constructive filing date (priority date) of the senior party, or that she was first to conceive and diligently reduced the invention to practice, starting from a date prior to reduction to practice by the senior party.

Patent Law > Claims & Specifications > Description Requirement > Written Description Versus Enablement
[HN2]Written description is a question of fact, judged from the perspective of one of ordinary skill in the art as of the relevant filing date. Enablement is a question of law involving underlying factual inquiries.

Patent Law > Jurisdiction & Review > Standards of Review > General Overview
[HN3]The United States Court of Appeals for the Federal Circuit applies the standards of the Administrative Procedure Act in reviewing decisions of the Board of Patent Appeals and Interferences. Accordingly, the Federal Circuit will set aside actions of the Board if they are arbitrary, capricious, an abuse of discretion, or otherwise not in accordance with law, and it sets aside factual findings that are unsupported by substantial evidence.

Patent Law > Jurisdiction & Review > Standards of Review > De Novo Review
[HN4]The United States Court of Appeals for the Federal Circuit reviews questions of law de novo.

Patent Law > Jurisdiction & Review > Standards of Review > Substantial Evidence
[HN5]Substantial evidence is defined as that which a reasonable person might accept as adequate to support a conclusion. It requires an examination of the record as a whole, taking into account both the evidence that justifies and detracts from an agency's opinion. An agency decision can be supported by substantial evidence, even where the record will support several reasonable but contradictory conclusions.

Patent Law > Claims & Specifications > General Overview
[HN6]A patent need not teach, and preferably omits, what is well known in the art.

Patent Law > Claims & Specifications > Description Requirement > General Overview
[HN7]The United States Court of Appeals for the Federal Circuit's precedent clearly establishes that the patent applicant must convey to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention.

Patent Law > Claims & Specifications > Description Requirement > General Overview
[HN8]No length requirement exists for a disclosure to adequately describe an invention.

Patent Law > Claims & Specifications > Description Requirement > General Overview
[HN9]The United States Court of Appeals for the Federal Circuit holds, in accordance with its prior case law, that (1) examples are not necessary to support the adequacy of a written description (2) the written description standard may be met even where actual reduction to practice of an invention is absent; and (3) there is no per se rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure.

Patent Law > Claims & Specifications > Description Requirement > General Overview
[HN10]A claim will not be invalidated on 35 U.S.C.S. § 112 grounds simply because the embodiments of the specification do not contain examples explicitly covering the full scope of the claim language. That is because the patent specification is written for a person of skill in the art, and such a person comes to the patent with the knowledge of what has come before. Placed in that context, it is unnecessary to spell out every detail of the invention in the specification; only enough must be included to convince a person of skill in the art that the inventor possessed the invention and to enable such a person to make and use the invention without undue experimentation.

Patent Law > Claims & Specifications > Description Requirement > General Overview
[HN11]The written description requirement implements the principle that a patent must describe the technology that is sought to be patented; the requirement serves both to satisfy the inventor's obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed.

Patent Law > Claims & Specifications > Description Requirement > General Overview
[HN12]Although reduction to practice ordinarily provides the best evidence that an invention is complete, it does not follow that proof of reduction to practice is necessary in every case. Thus, to the extent that written

description requires a showing of possession of the invention, caselaw makes clear that an invention can be complete even where an actual reduction to practice is absent. The logical predicate of possession is, of course, completeness.

Patent Law > Claims & Specifications > Description Requirement > General Overview

[HN13]It is the binding precedent of the United States Court of Appeals for the Federal Circuit that judicial precedent does not set forth a per se rule that whenever a claim limitation is directed to a macromolecular sequence, the specification must always recite the gene or sequence, regardless of whether it is known in the prior art. Thus, when the prior art includes the nucleotide information, precedent does not set a per se rule that the information must be determined afresh. Rather, the descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the state of knowledge in the field and differences in the predictability of the science.

Patent Law > Claims & Specifications > Description Requirement > General Overview

[HN14]According to the United States Court of Appeals for the Federal Circuit, the written description requirement states that the patentee must describe the invention; it does not state that every invention must be described in the same way. As each field evolves, the balance also evolves between what is known and what is added by each inventive contribution.

Patent Law > Claims & Specifications > Description Requirement > General Overview

[HN15]The United States Court of Appeals for the Federal Circuit holds that where accessible literature sources clearly provided, as of the relevant date, genes and their nucleotide sequences, satisfaction of the written description requirement does not require either the recitation or incorporation by reference (where permitted) of such genes and sequences.

COUNSEL: John P. Isacson, Heller Ehrman LLP, of Washington, DC, argued for appellants. With him on the brief was Paul M. Booth.

Robert G. McMorrow, Jr., Connolly Bove Lodge & Hutz LLP, of Wilmington, Delaware, argued for appellee.

JUDGES: Before GAJARSA, Circuit Judge, ARCHER, Senior Circuit Judge and DYK, Circuit Judge.

OPINION BY: GAJARSA

OPINION

[*1359] GAJARSA, Circuit Judge.

This is an appeal from a decision of the Board of Patent Appeals and Interferences ("Board") in Interference No. 105,187, declared on December 24, 2003, between Falkner *et al.*, U.S. Patent No. 5,770,212 ("the Falkner '212 patent") and Inglis *et al.*, U.S. Application Serial No. 08/459,040 ("the Inglis '040 application"). The Administrative Patent Judge (APJ) designated Inglis as the senior party. On December 29, 2004, the Board issued a final decision, holding that Falkner could not antedate Inglis' September 25, 1990 priority date, and entered judgment against Falkner on the sole count of the interference. It ordered that Falkner was not entitled to claims 1-19 of the Falkner '212 patent. It further [**2] ordered that Inglis was entitled to claims 9, 10, 29 and 30 of the '040 application. Falkner filed a timely notice of appeal. This Court has jurisdiction pursuant to 28 U.S.C. § 1295(a)(4)(A) and 35 U.S.C. §§ 141 and 142. For the reasons discussed below, we affirm the judgment of the Board.

I. BACKGROUND

A. The Invention

Some vaccines against a virus (the "target virus") incorporate harmless fragments of the target virus's genetic material into a second virus, called a "viral vector." When a person is vaccinated, the viral vector produces harmless fragments of the target virus, ultimately conferring immunity against it. To prevent the viral vector from itself causing a harmful infection in [*1360] the inoculee, it must be attenuated. Attenuation is achieved by deleting or inactivating one or more genes responsible for the vector's growth and infectiousness. However, because the vaccine is produced by essentially "growing" the vector virus (accompanied by its inserted target virus gene), attenuation makes it difficult to manufacture the vaccine. The traditional solution to this problem has been to inactivate genes [**3] known as "inessential" genes. With inessential genes inactivated, the viral vector is substantially less pathogenic. At the same time, because the vector virus can still fully reproduce itself, albeit more slowly, the vaccine can be produced in commercial quantities. However, the traditional approach carried a disadvantage, namely the

risk that the vector virus, though attenuated, could still cause a harmful infection in the inoculee.

The inventors discovered a way of making vaccines safer by deleting or inactivating an essential, rather than an inessential, gene from the viral vector's genome, while at the same time solving the production problem by growing the vaccines in cells that were complementarily modified to produce the absent essential viral gene product "on behalf of" the vector virus. Thus, the modified vector virus could be readily grown in these complementarily-modified cells, but not in other cells, such as those of an inoculee.

This approach is applicable to many different kinds of vector viruses, e.g., adenoviruses, herpesviruses, poxviruses and retroviruses. The subject matter of this interference, however, is directed specifically to vaccines in which the vector [**4] virus is a poxvirus. For many vector viruses, there is a risk that vectors that have been attenuated in essential genes can "swap" genes with the host cell genome, thereby reacquiring their deleted genes and reverting to wild-type virus. This risk can be minimized through the use of viruses that are "cytoplasmic", meaning that they are unlikely to enter the cell nucleus. Because a cell's genes are located in the nucleus, cytoplasmic viruses such as poxvirus cannot swap genes with the cell genome and possibly revert to a virulent wild-type virus.

B. Defining the Count and Assigning Priority

The sole count of the interference was either "[a] vaccine according to Claim 1 of Falkner's 5,770,212 patent or a vaccine according to Claim 29 of Inglis' 08/459,040 application." Claim 29 of the Inglis '040 application reads:

> A vaccine comprising a pharmaceutically acceptable excipient and an effective immunizing amount of a mutant virus, wherein said mutant virus is a mutant poxvirus and has a genome which has an inactivating mutation in a viral gene, said viral gene being essential for the production of infectious new virus particles, wherein said mutant virus is able to cause [**5] production of infectious new virus particles in a complementing host cell gene expressing a gene which complements said essential viral gene, but is unable to cause production of infectious new virus particles when said mutant virus infects a host cell other than a complementing host cell; for prophylactic or therapeutic use in generating an immune response in a subject.

(emphasis added)

Claim 1 of the Falkner '212 patent reads:

> A vaccine comprising (a) a defective poxvirus that lacks a function imparted by an essential region of its parental poxvirus, wherein (i) said defective poxvirus comprises a DNA polynucleotide encoding an antigen and said DNA polynucleotide is under transcriptional control of a promoter, and (ii) the function can [*1361] be complemented by a complementing source; and (b) a pharmaceutically acceptable carrier.

The Administrative Patent Judge (APJ) designated claims 1-19 of the Falkner '212 patent and claims 9,10, 29, and 30 of the Inglis '040 application as corresponding to the interference count. [1] Both parties sought the benefit of earlier-filed applications to establish dates of constructive reduction to practice. [2] The ALJ accorded the [**6] Inglis '040 application (filed June 2, 1995) the benefit of several earlier-filed applications, dating back to September 25, 1990. [3] Likewise, the APJ accorded the Falkner '212 patent (issued June 23, 1998 from an application filed February 21, 1997) the benefit of earlier-filed applications, but these dated back only to April 29, 1994. [4] Consequently, the APJ designated Inglis as the senior party.

1 Inglis's claim 29 is his broadest claim, directed to poxvirus; and claim 30, which depends on claim 29, is a poxvirus vaccine for mammalian subjects. Claim 9 is directed to poxvirus but contains some additional limitations unrelated to the type of virus used; claim 10 depends on claim 9 and is directed to a single species of poxvirus, namely vaccinia virus. Falkner's claims 2-10 depend on claim 1. Falkner claim 10 is directed to a method of producing the vaccine of claim 1, and the remaining method claims depend thereon.

2 [HN1]Priority in an interference goes to the first to invent, but a rebuttable presumption exists that the inventors made their inventions in the chronological order of their effective filing dates, namely that the senior party invented first, see 37 C.F.R. § 1.657(a) (2004), and the junior party bears the burden of proving otherwise, see § 1.657(b), such as by proving that she actually

reduced the invention to practice before the constructive filing date (priority date) of the senior party, or that she was first to conceive and diligently reduced the invention to practice, starting from a date prior to reduction to practice by the senior party. See 35 U.S.C. § 102(g) (2000). Falkner sought to rely, in part, on an alleged date of conception and beginning of reasonable diligence: April 27, 1994.

On September 13, 2004, the "600" rules expired in favor of new rules found at 37 C.F.R. § 41.100 et seq. However, the Board correctly chose to decide the matter under the old rules, given the parties' reliance on them in filing all motions, oppositions, and replies in the case, which were completed before the new rules took effect. See Singh v. Brake, 222 F.3d 1362, 1371 (Fed. Cir. 2000) (applying a new procedural rule if and only if it did not affect the parties' reliance interests).

[**7]

3 The Inglis priority applications were U.S. Application Serial No. 08/384,963 ("the Inglis '963 application"), filed February 7, 1995; U.S. Application Serial No. 08/030,073 ("the Inglis '073 application"), filed May 20, 1993; WO/92/05263, PCT/GB91/01632 ("the Inglis PCT application"), filed September 23, 1991, published in English on April 2, 1992; GB 9104903.1 ("the Inglis 1991 British application"), filed March 8, 1991; and GB 9020799.4 ("the Inglis 1990 British application"), filed September 25, 1990. The Inglis '040 application is a continuation in part of the '963 application, which was in turn a continuation of the Inglis '073 application. The '073 application corresponded to the Inglis PCT application. The Inglis PCT application claimed priority to, and was essentially identical to, the Inglis 1990 and 1991 British applications.

4 The Falkner priority applications were U.S. Application Serial No. 08/616,313 ("the Falkner '313 application") filed March 14, 1996; and U.S. application Serial No. 08/235,392 ("the Faulkner '392 application"), filed April 29, 1994.

C. Board Decision

The specifications of all of Inglis' earlier applications were similar. Although [**8] they focused on herpesvirus vectors, they contained several passages related to poxvirus-based vaccines. Because Falkner believed that these passages did not adequately describe and enable the poxvirus invention, he challenged both Inglis' entitlement to priority as to the count and the patentability of Inglis' corresponding claims. Falkner brought these challenges [*1362] in three closely-related preliminary motions before the Board. In each, as the moving party, Falkner carried the burden of proof by a preponderance of the evidence. See 37 C.F.R. § 1.637(a); see also Kubota v. Shibuya, 999 F.2d 517, 520 n.2 (Fed. Cir. 1993) (explaining that "[t]he term 'burden of proof' . . . means the burden to establish the proposition at issue by a preponderance of the evidence").

Falkner brought his first preliminary motion pursuant to 37 C.F.R. § 1.633(a), [5] arguing that the claims in Inglis's involved ('040) application that corresponded to the count were unpatentable because they failed to meet the written description requirement of 35 U.S.C. § 112. In support of his argument, he stated, inter alia, that (1) the specification of Inglis's '040 application [**9] did not identify any essential genes in poxvirus or describe the inactivation of such genes, (2) vaccines based on vaccinia (a type of poxvirus) had not yet been produced, and (3) the bulk of the Inglis specification was directed not to poxviruses but to herpesviruses. The Board denied Falkner's motion, based in part on his failure to address the perceived shortcomings of the '040 claims in light of the specification.

5 On September 13, 2004, the "600" rules expired in favor of new rules found at 37 C.F.R. § 41.100 et seq. However, the Board correctly decided the matter under the old rules, given the parties' reliance on them in filing all motions, oppositions, and replies in the case, which were completed before the new rules took effect. See Singh v. Brake, 222 F.3d 1362, 1371 (Fed. Cir. 2000) (applying a new procedural rule if and only if it did not affect the parties' reliance interests); see also Brown v. Barbacid, 436 F.3d 1376, 1379 n.1 (Fed. Cir. 2006) (holding that the Board did not err in applying the old rules "under which this case was decided").

Second, Falkner moved pursuant to 37 C. [**10] F.R. §§ 1.633(g) & 1.637(g) to deny Inglis the priority benefit of his earlier applications, arguing that they did not sufficiently describe and enable the claims in question. [6] Falkner argued that without the benefit of these applications Inglis would be unable to establish constructive reduction to practice earlier than Falkner. Falkner would win priority as to the count, and Inglis' corresponding claims would be unpatentable. In support of his motion, Falkner alleged deficiencies in Inglis' benefit specifications similar to those raised in his first motion. The Board carefully articulated the legal standard, correctly explaining that "benefit with respect

to priority in an interference is granted with respect to counts not claims" and that "[a]ll that is necessary for a party to be entitled to benefit of an earlier filed application for priority purposes is compliance with 35 U.S.C. § 112 with respect to at least one embodiment within the scope of the count." Board Op. at 7 (citing Hunt v. Treppschuh, 523 F.2d 1386, 1389 (CCPA 1975) (holding that where a "parent application is relied upon as a prior constructive reduction to [**11] practice[,] . . . . the § 112, first paragraph requirements need only be met for an embodiment within the count")). After careful review of the record, the Board held that Falkner had failed to meet his burden of proof.

> 6 Falkner did not argue lack of enablement with respect to the Inglis '963 patent because he believed that the teachings of the Falkner '392 patent, filed in 1994, would have enabled the subsequent '963 patent.

Third, Falkner moved for judgment pursuant to 37 C.F.R. § 1.633(a) that the claims in Inglis' involved ('040) application that corresponded to the count were anticipated and therefore unpatentable. He argued that because Inglis' earlier applications had failed to adequately describe and enable the full scope of his current claims, the current claims could not be accorded the benefit of 35 U.S.C. § 120 for the [*1363] purpose of antedating patent-defeating prior art. [7] The Board explained that 35 U.S.C. §§ 119 & 120 require benefit applications to comply with § 112, first paragraph, with respect to the full scope of what a party now claims, rather than with respect to merely [**12] one embodiment within the scope of the interference count. After carefully considering the written description and enablement issues, the Board denied the motion. As a result of the denial of Falkner's several motions, Inglis remained the senior party, and the Board ordered judgment as to the subject matter of the count in favor of Inglis.

> 7 Here, Falkner points to his own U.S. Pat. No. 5,766,882 ("the '882 patent'), issued in March 1995, as the patent-defeating prior art.

D. Issue and Standard of Review

On appeal, Falkner essentially reiterates the arguments that he made before the Board. While we recognize that each of these three arguments is distinct, they are nonetheless all related, and under the facts of this particular case, we need only to resolve the following common issue: whether the Inglis benefit applications adequately describe and enable a poxvirus-based vaccine. Falkner also argues that the Board committed other errors, such as initially designating Inglis as the senior party and failing to afford Falkner an opportunity for briefing prior to making this designation. These arguments lack merit, and we shall not further discuss them. We turn, therefore, [**13] to the central issues in this case: written description and enablement.

[HN2]Written description is a question of fact, judged from the perspective of one of ordinary skill in the art as of the relevant filing date. See Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 1563-64 (Fed. Cir. 1991). Enablement is a question of law involving underlying factual inquiries. See Genentech, Inc. v. Novo Nordisk A/S, 108 F.3d 1361, 1365 (Fed. Cir. 1997); see also In re Wands, 858 F.2d 731, 737 (Fed. Cir. 1988) (holding that whether undue experimentation is required is a "conclusion reached by weighing many factual considerations. . . . includ[ing] (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.").

[HN3]This court applies the standards of the Administrative Procedure Act ("APA") in reviewing decisions of the Board. See Dickinson v. Zurko, 527 U.S. 150, 152, 119 S. Ct. 1816, 144 L. Ed. 2d 143 (1999) [**14] (holding that 5 U.S.C. § 706 governs our review of PTO appeals). Accordingly, we will set aside actions of the Board if they are arbitrary, capricious, an abuse of discretion, or otherwise not in accordance with law, and we set aside factual findings that are unsupported by substantial evidence. See In re McDaniel, 293 F.3d 1379, 1382 (Fed. Cir. 2002) (citing 5 U.S.C. § 706); see also In re Sullivan, 362 F.3d 1324, 1326 (Fed. Cir. 2004) (substantial evidence review of factual findings). [HN4]We review questions of law de novo. See Rapoport v. Dement, 254 F.3d 1053, 1058 (Fed. Cir. 2001).

[HN5]Substantial evidence is defined as that which a reasonable person might accept as adequate to support a conclusion. See In re Zurko, 258 F.3d 1379, 1384 (Fed. Cir. 2001). It requires an examination of the record as a whole, taking into account both the evidence that justifies and detracts from an agency's opinion. See In re Gartside, 203 F.3d 1305, 1312 (Fed. Cir. [*1364] 2000). An agency decision can be supported by substantial evidence, even where the record will support several [**15] reasonable but contradictory conclusions. See id.; see also In re Jolley, 308 F.3d 1317, 1320 (Fed. Cir. 2002).

II. DISCUSSION

A. Contents of the Inglis Priority Applications

The claims that correspond to the count of the interference are directed to a novel type of vaccine that is comprised of a "vector virus" in the poxvirus family. Conceptually, poxviruses are a "subgenus" of viruses that includes the "species" vaccinia. All of the prior Falkner applications described poxvirus vaccine vectors in detail, and to the exclusion of other types of vaccine vectors (e.g., herpesvirus vaccine vectors). These applications provided five detailed working examples regarding the preparation and use of vaccines from defective poxviruses. They also described the use of a particular species of poxvirus vaccine vector, namely vaccinia virus.

In contrast, the Inglis applications described vaccine vectors in general, and then focused on the subgenus of herpesviruses, for which they provided a detailed example. Nevertheless, at least three passages discussed the poxvirus invention and specifically mentioned "vaccinia virus." [8] For example, after introducing the concept [**16] of vaccine vectors, the specification states that "[t]ypically members of the pox virus family, e.g. vaccinia virus, are used as vaccine vectors." [9] The specification later discusses the deletion of essential genes from vaccine vector genomes, noting that the "invention can be applied to any virus where one or more essential gene(s) can be identified and deleted from or inactivated within the virus genome" (emphasis added). Moreover, it provides that "the virus may comprise an orthopox virus, for example, vaccinia virus, which may comprise a heterologous sequence encoding an immunogen derived from a pathogen." Finally, it reads:

> For example vaccinia virus, a poxvirus, can carry and express genes from various pathogens, and it has been demonstrated that these form effective vaccines when used in animal experimental systems. The potential for use in humans is vast, but because of the known side effects associated with the widespread use of vaccinia as a vaccine against smallpox, there is reluctance to use an unmodified vaccine in humans. There have been attempts to attenuate vaccinia virus by deleting non-essential genes such as the vaccinia growth factor gene. . . However, [**17] such attenuated viruses can still replicate in vivo, albeit at a reduced level. No vaccinia virus with a deletion in an essential gene has yet been produced, but such a virus, deleted in an essential gene as described above, with its complementing cell for growth, would provide a safer version of this vaccine.

8 We recognize that the Inglis applications do not describe any actual reduction to practice of a poxvirus vaccine. See Carroll Declaration (stating that the '040 application did not contain any discussion of the "actual creation of the recited 'mutant poxvirus'" and that the application in fact stated "that a vaccinia virus with a deletion in an essential gene had not been produced."). As we discuss below, however, an actual reduction to practice is unnecessary to satisfy the written description requirement.

9 Because of the substantial similarity in the specifications of all of the Inglis benefit applications, we shall refer in this opinion to representative passages from the earliest of the applications, the Inglis 1990 British application.

The application provides a detailed example of an embodiment that comprised not a poxvirus, but a [**18] herpesvirus, including [*1365] the identity of the deleted essential sequences therein. Nevertheless, for the reasons discussed below, we find no error in the Board's determinations on the adequacy of written description and enablement in the various Inglis disclosures.

B. Enablement

Because the adequacy of the disclosure is judged from the perspective of one of ordinary skill in the art, we start our review of the Board's decision by noting that the parties stipulated to a high level of skill in the art. They defined the skilled artisan as having 5-10 years experience creating recombinant poxvirus, as being familiar with the poxvirus literature, the use of poxvirus as a vector for the expression of heterologous genes, and having the "needed technical skill to practice the experimentation described in the scientific literature relating to recombinant virus, including poxvirus." The Board agreed with the parties' stipulation as to level of skill.

The Board did not err in finding Inglis' claims to be enabled as a matter of law, in light of its articulated underlying factual findings. In support of its conclusion, it noted that "there is extensive disclosure of the selection of an essential [**19] gene, its deletion or inactivation and the production of a mutated virus with said deleted or inactivated gene, albeit for herpesvirus." Moreover, because the differences between the herpesviruses and poxviruses were well known, this would have aided the person of ordinary skill in the art in her application of the lessons of the herpesvirus example in the construction of poxvirus vaccines. The Board observed that "the mere fact that the experimentation

may have been difficult and time consuming does not mandate a conclusion that such experimentation would have been considered to be 'undue' in this art. Indeed, great expenditures of time and effort were ordinary in the field of vaccine preparation." Thus, the Board found the Inglis applications to be enabling.

Reviewing the Board's legal conclusion of enablement, as based on its underlying findings of fact, we cannot say that the Board erred. With respect to a skilled artisan's ability to identify "essential" poxvirus genes, as discussed below we note that there was undisputed testimony that as of the time of filing of the earliest Inglis application publications in professional journals had disclosed the DNA sequence of the poxvirus [**20] genome along with the locations of the "essential regions." The person of ordinary skill in the art would clearly have possessed such knowledge, and given the ready accessibility of the journals, the absence of incorporation by reference is not problematic. Indeed, [HN6]"[a] patent need not teach, and preferably omits, what is well known in the art." Spectra-Physics, Inc. v. Coherent, Inc., 827 F.2d 1524, 1534 (Fed. Cir. 1987).

C. Written Description

On appeal to this court, Falkner essentially reargues the positions on written description that he took before the Board. Although the Board erred in its articulation of the written description standard, that error is harmless. The Board held that "an actual possession standard is not required." (emphasis added). But [HN7]our precedent clearly establishes that "[t]he applicant must . . . convey to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention." Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 1563-64 (Fed. Cir. 1991). Nonetheless, we conclude there is no need for remand because the undisputed testimony supports the Board's ultimate conclusion.

As noted [**21] above, the Board found several passages in the Inglis '040 application (and in the benefit applications) that were directed to poxvirus. [HN8]No length requirement exists for a disclosure to adequately [*1366] describe an invention. See In re Hayes Microcomputer Prods., Inc. Patent Litig., 982 F.2d 1527, 1534 (Fed. Cir. 1992) ("[T]he adequacy of the description of an invention depends on its content in relation to the particular invention, not its length."). Furthermore, the testimony of Falkner's expert, Dr. Boursnell, established that the articles describing essential genes for poxvirus were well-known in the art. Dr. Boursnell testified that "the skilled person would have been readily able to choose an essential vaccinia gene" based on references that have been publicly available since 1990. The testimony of Inglis' expert, Dr. Carroll, did not refute this claim.

The parties also dispute several aspects of our law of written description, which we now address. We conclude that the Board applied correct law. Specifically, [HN9]we hold, in accordance with our prior case law, that (1) examples are not necessary to support the adequacy of a written description (2) the written description [**22] standard may be met (as it is here) even where actual reduction to practice of an invention is absent; and (3) there is no per se rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure.

1. Examples Are Not Required

First, it is clear that the absence of examples involving poxviruses in the Inglis applications does not render the written description inadequate. As we explained in LizardTech, Inc. v. Earth Resource Mapping, PTY, Inc.:

> [HN10]A claim will not be invalidated on section 112 grounds simply because the embodiments of the specification do not contain examples explicitly covering the full scope of the claim language. That is because the patent specification is written for a person of skill in the art, and such a person comes to the patent with the knowledge of what has come before. Placed in that context, it is unnecessary to spell out every detail of the invention in the specification; only enough must be included to convince a person of skill in the art that the inventor possessed the invention and to enable such a person to make and use the invention without undue experimentation. [**23]

424 F.3d 1336, 1345 (Fed. Cir. 2005) (citing Union Oil Co. v. Atl. Richfield Co., 208 F.3d 989, 997 (Fed. Cir. 2000); In re GPAC Inc., 57 F.3d 1573, 1579 (Fed. Cir. 1995)).

2. Actual Reduction to Practice Is Not Required

As we explained in Capon v. Eshhar, [HN11]"[t]he 'written description' requirement implements the principle that a patent must describe the technology that is sought to be patented; the requirement serves both to satisfy the inventor's obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed." 418 F.3d 1349, 1357 (Fed. Cir. 2005). The Board was correct, however, not to view as dispositive that Inglis had not actually produced a

poxvirus vaccine, [10] because an actual reduction to practice is not required for written description. [11] See [*1367] Univ. of Rochester v. G.D. Searle & Co., 358 F.3d 916, 926 (Fed. Cir. 2004) ("We of course do not mean to suggest that the written description requirement can be satisfied only by providing a description of an actual reduction to practice. [**24] Constructive reduction to practice is an established method of disclosure . . . ."). Rochester, moreover, is consistent with Supreme Court precedent. In the context of interpreting 35 U.S.C. § 102(b), the Court held that "[t]he word 'invention' must refer to a concept that is complete, rather than merely one that is 'substantially complete.'" Pfaff v. Wells Elecs., 525 U.S. 55, 66, 119 S. Ct. 304, 142 L. Ed. 2d 261 (1998). It then proceeded to make clear that [HN12]although "reduction to practice ordinarily provides the best evidence that an invention is complete. . . . it does not follow that proof of reduction to practice is necessary in every case." Id.(emphasis added). [12] Thus, to the extent that written description requires a showing of "possession of the invention," Capon, 418 F.3d at 1357 (emphasis added), Pfaff makes clear that an invention can be "complete" even where an actual reduction to practice is absent. [13] The logical predicate of "possession" is, of course, "completeness."

10 The Inglis specifications stated that "[n]o vaccinia virus with a deletion in an essential gene has yet been produced, but such a virus, deleted in an essential gene as described above, with its complementing cell for growth, would provide a safer version of this vaccine."

[**25]

11 The Board believed that Falkner's expert, Dr. Carroll, had premised his opinions on the misunderstanding that actual reduction to practice was required to prove written description, and it discredited his expert opinion.

12 Similarly, this court has carefully explained the relationship between written description and possession, explaining that a showing of possession is not necessarily sufficient to demonstrate the adequacy of written description. See, e.g., Enzo Biochem, Inc. v. Gen-Probe Inc., 296 F.3d 1316, 1330 (Fed. Cir. 2002) ("[P]roof of a reduction to practice, absent an adequate description in the specification of what is reduced to practice, does not serve to describe or identify the invention for purposes of § 112, P1. As with 'possession,' proof of a reduction to practice may show priority of invention or allow one to antedate a reference, but it does not by itself provide a written description in the patent specification.").

13 In contrast to reduction to practice, conception is a prerequisite to an adequate written description. See Fiers v. Sugano, 984 F.2d 1164, 1171 (Fed. Cir. 1993) ("[O]ne cannot describe what one has not conceived.").

[**26] 3. Recitation of Known Structure Is Not Required

Falkner argues, inter alia, that the Inglis specifications do not adequately describe the poxvirus invention, in light of Eli Lilly, because they do not describe the "essential regions" of any poxvirus. 119 F.3d 1559. We note, in addition, that Inglis did not attempt to incorporate by reference any literature that described the DNA sequence of the poxvirus genome and the locations of the "essential regions." However, [HN13]it is the binding precedent of this court that Eli Lilly does not set forth a per se rule that whenever a claim limitation is directed to a macromolecular sequence, the specification must always recite the gene or sequence, regardless of whether it is known in the prior art. See Capon, 418 F.3d at 1357 ("None of the cases to which the Board attributes the requirement of total DNA re-analysis, i.e., Regents v. Lilly, Fiers v. Revel, Amgen, or Enzo Biochem, require a re-description of what was already known."). Thus, "[w]hen the prior art includes the nucleotide information, precedent does not set a per se rule that the information must be determined [**27] afresh." Id. at 1358. Rather, we explained that:

> The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the [*1368] state of knowledge in the field and differences in the predictability of the science.

Id. at 1357.

Indeed, a requirement that patentees recite known DNA structures, if one existed, would serve no goal of the written description requirement. It would neither enforce the quid pro quo between the patentee and the public by forcing the disclosure of new information, nor would it be necessary to demonstrate to a person of

ordinary skill in the art that the patentee was in possession of the claimed invention. As we stated in Capon, [HN14]"[t]he 'written description' requirement states that the patentee must describe the invention; [**28] it does not state that every invention must be described in the same way. As each field evolves, the balance also evolves between what is known and what is added by each inventive contribution." Id. at 1358. Indeed, the forced recitation of known sequences in patent disclosures would only add unnecessary bulk to the specification. Accordingly [HN15]we hold that where, as in this case, accessible literature sources clearly provided, as of the relevant date, genes and their nucleotide sequences (here "essential genes"), satisfaction of the written description requirement does not require either the recitation or incorporation by reference [14] (where permitted) of such genes and sequences.

> 14 Here, the patentee did not attempt incorporation by reference. Where, of course, certain material that is not present in the specification is deemed nonessential to the satisfaction of the written description requirement, the issue of proper incorporation by reference vel non is irrelevant.

In conclusion, having reviewed the decision of the Board, we can discern no error in its conclusion that the disclosures relied upon by Inglis for priority purposes adequately [**29] described and enabled the invention directed to poxvirus, there being substantial evidence to support these findings. Consequently, we hold that the Board's award of priority to Inglis was proper.

AFFIRMED

No costs.

LEXSEE

Positive
As of: Aug 19, 2008

IN RE JOYCE A. CORTRIGHT

98-1258

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

165 F.3d 1353; 1999 U.S. App. LEXIS 729; 49 U.S.P.Q.2D (BNA) 1464

January 19, 1999, Decided

SUBSEQUENT HISTORY: [**1] Rehearing Denied April 20, 1999, Reported at: 1999 U.S. App. LEXIS 9001.

PRIOR HISTORY: Appealed from: U.S. Patent and Trademark Office Board of Patent Appeals and Interferences. (Serial No. 07/849,191).

DISPOSITION: AFFIRMED-IN-PART, REVERSED-IN-PART, AND REMANDED.

CASE SUMMARY:

PROCEDURAL POSTURE: Petitioner appealed from the decision of the U.S. Patent and Trademark Office Board of Patent Appeals in a case concerning rejection of certain claims on her patent application.

OVERVIEW: Petitioner appealed from the decisions of the United States Board of Patent Appeals sustaining a rejection of claims to her patent application. Petitioner's patent application concerned a method of treating baldness by applying a Bag Balm, a commercially available product used to soften cow udders, to the human scalp. The examiner rejected the claim under 35 U.S.C.S. § 101 as lacking utility. The court held that the board erred in suggesting that petitioner was required to prove the cause of resultant hair growth. It was not a requirement of patentability that an inventor correctly set forth, or even know, how or why an invention worked. However, the court did rule that on one particular claim, that the use requirement was not satisfied. Therefore, the judgment of the patent board was affirmed in part and reversed in part.

OUTCOME: The Board of Patent Appeals rejected petitioner's patent application. On appeal, the court reversed in part and affirmed in part because one of the claims did not satisfy the use requirement, but the court stated that on another claim, petitioner was did not need to how or why an invention worked.

LexisNexis(R) Headnotes

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN1]Whether making and using an invention would have required undue experimentation, and thus whether a disclosure is enabling under 35 U.S.C.S. § 112, is a legal conclusion based upon underlying factual inquiries.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN2]See 35 U.S.C.S. § 112.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN3]A lack of enablement rejection under 35 U.S.C.S. § 112, is appropriate where the written description fails to teach those in the art to make and use the invention as broadly as it is claimed without undue experimentation.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview

Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Utility Requirement > Fact & Law Issues
[HN4]Thus, an applicant's failure to disclose how to use an invention may support a rejection under either 35 U.S.C.S§ 112, for lack of enablement as a result of the specification's. Failure to disclose adequately to one ordinarily skilled in the art how to use the invention without undue experimentation, or 35 U.S.C.S. § 101 for lack of utility when there is a complete absence of data supporting the statements which set forth the desired results of the claimed invention.

Evidence > Inferences & Presumptions > Presumption of Regularity
Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Inequitable Conduct > General Overview
[HN5]The Patent and Trademark Office (PTO) cannot make this type of rejection, however, unless it has reason to doubt the objective truth of the statements contained in the written description. The PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Subject Matter > Processes > Computer Software & Mental Steps
[HN6]A specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of 35 U.S.C.S. § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.. The Patent and Trademark Office (PTO) may establish a reason to doubt an invention's asserted utility when the written description suggests an inherently unbelievable undertaking or involves implausible scientific principles.

Patent Law > Claims & Specifications > Claim Language > General Overview
Patent Law > U.S. Patent & Trademark Office Proceedings > Reissues > General Overview
[HN7]The Patent and Trademark Office applies to the verbiage of the proposed claims the broadest reasonable meaning of the words in their ordinary usage as they would be understood by one of ordinary skill in the art. It is axiomatic that, in proceedings before the Patent and Trademark Office, claims in an application are to be given their broadest reasonable interpretation consistent with the specification, and that claim language should be read in light of the specification as it would be interpreted by one of ordinary skill in the art.

Patent Law > Claims & Specifications > General Overview
[HN8]It is not a requirement of patentability that an inventor correctly set forth, or even know, how or why the invention works.

Patent Law > Claims & Specifications > Description Requirement > General Overview
[HN9]In order for a disclosure to be inherent the missing descriptive matter must necessarily be present in the application's specification such that one skilled in the art would recognize such a disclosure.

COUNSEL: Joseph B. Taphorn, of Poughkeepsie, New York, argued for appellant.

Scott A. Chambers, Associate Solicitor, U.S. Patent and Trademark Office, of Arlington, Virginia, argued for the appellee. With him on the brief were Nancy J. Linck, Solicitor, Albin F. Drost, Deputy Solicitor, and Linda Moncys Isacson, Associate Solicitor.

JUDGES: Before MAYER, Chief Judge, NEWMAN, and RADER, Circuit Judges.

OPINION BY: MAYER

OPINION

[*1355] MAYER, *Chief Judge.*

Joyce A. Cortright appeals the September 23 and November 28, 1997, decisions of the United States Board of Patent Appeals and Interferences sustaining the rejection of claims 1 and 15 of patent application Serial No. 07/849,191 under 35 U.S.C. § 112, P 1 (1994). Because the board erred with respect to claim 1 but not claim 15, we affirm-in-part, reverse-in-part, and remand.

Background

Cortright's patent application, filed in 1992, concerns a method of treating baldness by applying Bag Balm, (R) a commercially available product used to sof-

ten cow udders, to human scalp. [**2] Claims 1 and 15 are the only claims on appeal. Claim 1 recites a method of "treating scalp baldness with an antimicrobial to restore hair growth, which comprises rubbing into the scalp the ointment wherein the active ingredient 8-hydroxyquinoline sulfate 0.3% is carried in a petrolatum and lanolin base." Claim 15 recites a method of "offsetting the effects of lower levels of a male hormone being supplied by arteries to the papilla of scalp hair follicles with the active agent 8-hydroxy-quinoline sulfate to cause hair to grow again on the scalp, comprising rubbing into the scalp the ointment having the active agent 8-hydroxyquinoline sulfate 0.3% carried in a petrolatum and lanolin base so that the active agent reaches the papilla."

The examiner rejected the claims under 35 U.S.C. § 101 (1994) as lacking utility. According to the examiner, Cortright's statements of utility, namely, her claims of treating baldness, are suspect because "baldness is generally accepted in the art as being incurable . . . ." The examiner, therefore, required clinical evidence to establish the claimed utility, which Cortright did not supply. Furthermore, with respect to claim 15's recitation of offsetting [**3] the effects of lower levels of a male hormone, Cortright "offered no proof that such an off-set occurs and has disclosed that this is only speculation." The examiner also rejected the claims under 35 U.S.C. § 102(a) (1994), arguing that the admitted prior art anticipates the claims because the written description discloses that Bag Balm (R) has been applied to human skin and the "scalp is the skin of the head." Cortright appealed these rejections to the Board of Patent Appeals and Interferences.

In its September 23, 1997, decision, the board reversed the section 101 rejection because the examiner did not set out sufficient reasons for finding Cortright's statements of utility incredible. It noted that "there is no per se requirement for clinical evidence to establish the utility of any invention" and the examples in Cortright's application are objective evidence. The board also reversed the section 102(a) rejection because although the prior art discloses the application of Bag Balm (R) to human skin, it does not disclose applying it to bald, human scalp.

Despite these reversals, Cortright did not prevail because the board found a new ground for rejecting the claims: that they are [**4] based on a non-enabling disclosure in violation of 35 U.S.C. § 112, P 1. The board found that Cortright's written description does not teach those of ordinary skill in the art how to make and use the claimed invention without undue experimentation because it "fails to provide any teachings as to the administration of Bag Balm (R) in a manner which (i) restores hair growth (claim 1), or (ii) 'offsets the effects of lower levels of male hormone being supplied by arteries to the papilla of scalp hair follicles' (claim 15)." The board explained that Example 1 does not show that applying a teaspoon of Bag Balm (R) to the scalp daily for about one month "restored hair growth" and that Examples 2 and 3 do not disclose the amount of Bag Balm (R) to apply or how to restore hair growth. With respect to claim 15, the board found that the written description "merely surmises that the active ingredient, 8-hydroxyquinoline sulfate, even reaches the papilla," which would not enable one of ordinary skill to use the claimed method. Finally, the board observed that the breadth of the claims and the unpredictable nature of the art of hair growth aggravated its finding that those of ordinary skill in [**5] the art would not be able to practice the invention without undue experimentation.

Cortright requested reconsideration, which the board denied in a November 28, 1997, [*1356] opinion. The board explained that claim 1 is not enabled because it claims "restoring hair growth," which the board interpreted as requiring the user's hair "to return to its original state," that is, a full head of hair. Thus, the board's rejection was not based on complete non-enablement, as the original decision had implied, but on the claim not being commensurate with the scope of the disclosure. With respect to claim 15, the board maintained its general non-enablement rejection, adding that "there is no evidence of record that the resultant hair growth is due to (i) the stimulation of the papilla, and (ii) the offsetting [of] the effects of lower male hormone which is supplied by arteries to the papilla, and not due to some other mechanism(s)." Cortright appeals.

Discussion

"[HN1]Whether making and using an invention would have required undue experimentation, and thus whether a disclosure is enabling under 35 U.S.C. § 112, P 1 (1994), is a legal conclusion based upon underlying factual inquiries." *Johns Hopkins* [**6] *Univ. v. Cellpro, Inc.*, 152 F.3d 1342, 1354, 47 U.S.P.Q.2D (BNA) 1705, 1713 (Fed. Cir. 1998). Utility is a factual issue, which we review for clear error. *See Cross v. Iizuka*, 753 F.2d 1040, 1044 n.7, 224 U.S.P.Q. (BNA) 739, 742 n.7 (Fed. Cir. 1985); *see also In re Zurko*, 142 F.3d 1447, 1449, 46 U.S.P.Q.2D (BNA) 1691, 1693 (Fed. Cir.), *cert. granted*, 119 S. Ct. 401 (1998).

[HN2]Section 112, P 1 provides:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best

> mode contemplated by the inventor of carrying out his invention.

35 U.S.C. § 112, P 1. [HN3]A lack of enablement rejection under section 112, P 1 is appropriate where the written description fails to teach those in the art to make and use the invention as broadly as it is claimed without undue experimentation. *See In re Vaeck*, 947 F.2d 488, 495-96, 20 U.S.P.Q.2D (BNA) 1438, 1444 (Fed. Cir. 1991).

This rejection takes several forms. The PTO will make a scope [**7] of enablement rejection where the written description enables something within the scope of the claims, but the claims are not limited to that scope. *See* Manual of Patent Examining Procedures ("M.P.E.P.") § 706.03(c), form P 7.31.03 (Rev. 3, July 1997). This type of rejection is marked by language stating that the specification does not enable one of ordinary skill to use the invention commensurate with the scope of the claims. On the other hand, if the written description does not enable any subject matter within the scope of the claims, the PTO will make a general enablement rejection, stating that the specification does not teach how to make or use the invention. *See* M.P.E.P. § 706.03(c), form P 7.31.02.

If the written description fails to illuminate a credible utility, the PTO will make both a section 112, P 1 rejection for failure to teach how to use the invention and a section 101 rejection for lack of utility. *See* M.P.E.P. § 706.03(a), form P 7.05.04. This dual rejection occurs because "the how to use prong of section 112 incorporates as a matter of law the requirement of 35 U.S.C. § 101 that the specification disclose as a matter of fact a practical utility [**8] for the invention." *In re Ziegler*, 992 F.2d 1197, 1200, 26 U.S.P.Q.2D (BNA) 1600, 1603 (Fed. Cir. 1993). [HN4]Thus, an applicant's failure to disclose how to use an invention may support a rejection under either section 112, P 1 for lack of enablement as a result of "the specification's . . . failure to disclose adequately to one ordinarily skilled in the art 'how to use' the invention without undue experimentation," or section 101 for lack of utility "when there is a complete absence of data supporting the statements which set forth the desired results of the claimed invention." *Envirotech Corp. v. Al George, Inc.*, 730 F.2d 753, 762, 221 U.S.P.Q. (BNA) 473, 480 (Fed. Cir. 1984); *see also In re Brana*, 51 F.3d 1560, 1564 n.12, 34 U.S.P.Q.2D (BNA) 1436, 1439 n.12 (Fed. Cir. 1995) (The "absence of utility can be the basis of a rejection under both 35 U.S.C. § 101 and § 112, P 1."); *In re Fouche*, 58 C.C.P.A. 1086, 439 F.2d 1237, 1243, 169 U.S.P.Q. (BNA) 429, 434 (CCPA 1971) ("If [*1357] [certain] compositions are in fact useless, appellant's specification cannot have taught how to use them.").

[HN5]The PTO cannot make this type of rejection, however, unless it has reason to doubt the objective truth of the statements contained [**9] in the written description. *See Brana*, 51 F.3d at 1566, 34 U.S.P.Q.2D (BNA) at 1441 ("The PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility.") (citations omitted); *In re Marzocchi*, 58 C.C.P.A. 1069, 439 F.2d 220, 223, 169 U.S.P.Q. (BNA) 367, 369 (CCPA 1971) [HN6]("[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support."). The PTO may establish a reason to doubt an invention's asserted utility when the written description "suggests an inherently unbelievable undertaking or involves implausible [**10] scientific principles." *Brana*, 51 F.3d at 1566, 34 U.S.P.Q.2D (BNA) at 1441; *see also In re Eltgroth*, 57 C.C.P.A. 833, 419 F.2d 918, 164 U.S.P.Q. (BNA) 221 (CCPA 1970) (control of aging process). Treating baldness was once considered an inherently unbelievable undertaking. *See In re Ferens*, 57 C.C.P.A. 733, 417 F.2d 1072, 1074, 163 U.S.P.Q. (BNA) 609, 611 (CCPA 1969); *In re Oberweger*, 28 C.C.P.A. 749, 115 F.2d 826, 829, 47 U.S.P.Q. (BNA) 455, 458 (CCPA 1940).

Since then, however, treatments for baldness have gained acceptance. Rogaine (R) (minoxidil) and Propecia (R) are recognized as effective in treating baldness. *See* Doug Levy, *FDA Approves New Treatment for Males Fighting Baldness*, USA Today, Dec. 23, 1997, at A1; *Pharmaceutical Companies Are Brushing up on Hair-Restorers Medicine*, Los Angeles Times, Jun. 6, 1996, at D12. In addition, the PTO has granted approximately one hundred patents on methods of treating baldness. Some of these patents disclose applying an electric current to the scalp, *see, e.g.*, U.S. Pat. No. 5,800,477, whereas others teach ingesting substances orally or applying a salve of some kind to the scalp, *see, e.g.*, U.S. Pat. No. 5,777,134. Some patents disclose the [**11] active ingredient in chemical terms. *See, e.g.*, U.S. Pat. No. 5,777,134 (5 alpha-reductase inhibitor); U.S. Pat. No. 5,767,152 (cyanocarboxylic acid derivatives); U.S. Pat. No. 4,139,619 (formula for minoxidil). Other patents,

however, disclose baldness remedies made from more mundane materials, such as Dead Sea mud (U.S. Pat. No. 5,679,378); emu oil (U.S. Pat. No. 5,744,128); potato peelings and lantana leaves (U.S. Pat. No. 5,665,342); and vitamin D3 and aloe (U.S. Pat. No. 5,597,575). [1]

> 1 *See also* U.S. Pat. No. 5,674,510 (salve of garlic powder, brewer's yeast, grapefruit juice, acetic acid, and kelp), U.S. Pat. No. 5,750,108 (salves of tea tree oil; chlorine dioxide and acidic solution; saw palmetto berry extract), U.S. Pat. No. 5,695,748 (salves of sage, aloe, and nettles; castor oil, shea butter, wheat germ oil, and white iodine); U.S. Pat. No. 5,494,667 (salve of pine extract and bamboo extract or Japanese apricot).

Claim 1

With respect to claim 1, the examiner made a lack of utility rejection [**12] under section 101 arguing that the asserted statements of utility were incredible in light of Cortright's failure to prove utility with clinical evidence. The board first appeared to make a generic enablement rejection under section 112, P 1, focusing on "the lack of any teachings or guidance as to how to perform the claimed methods and the unpredictable nature of the art of restoring hair growth." Upon reconsideration, however, the board clarified that its rejection pertained to scope. It took the position that the broadest interpretation of "restore hair growth" requires the application of Bag Balm (R) to "return" the user's hair "to its original state," that is, a full head of hair. Because Cortright's written description discloses results of only "three times as [*1358] much hair growth as two months earlier," "filling-in some," and "fuzz," the board reasoned, it does not support the breadth of the claims.

Although the PTO must give claims their broadest reasonable interpretation, this interpretation must be consistent with the one that those skilled in the art would reach. *See In re Morris*, 127 F.3d 1048, 1054, 44 U.S.P.Q.2D (BNA) 1023, 1027 (Fed. Cir. 1997) ("[HN7]The PTO applies to the verbiage [**13] of the proposed claims the broadest reasonable meaning of the words in their ordinary usage as they would be understood by one of ordinary skill in the art . . . ."); *In re Bond*, 910 F.2d 831, 833, 15 U.S.P.Q.2D (BNA) 1566, 1567 (Fed. Cir. 1990) ("It is axiomatic that, in proceedings before the PTO, claims in an application are to be given their broadest reasonable interpretation consistent with the specification, . . . and that claim language should be read in light of the specification *as it would be interpreted by one of ordinary skill in the art.*") (emphasis added); *see also* M.P.E.P. § 2111.01 ("The words of a claim . . . must be read as they would be interpreted by those of ordinary skill in the art."). Prior art references may be "indicative of what all those skilled in the art generally believe a certain term means . . . [and] can often help to demonstrate how a disputed term is used by those skilled in the art." *Vitronics Corp. v. Conceptronic, Inc.*, 90 F.3d 1576, 1584, 39 U.S.P.Q.2D (BNA) 1573, 1578-79 (Fed. Cir. 1996). Accordingly, the PTO's interpretation of claim terms should not be so broad that it conflicts with the meaning given to identical terms in other patents from [**14] analogous art. *Cf. Morris*, 127 F.3d at 1056, 44 U.S.P.Q.2D (BNA) at 1029 (approving the board's definition of claim terms consistent with their definitions in CCPA cases).

The PTO's construction of "restore hair growth" in the present case is inconsistent with its previous definitions. U.S. Pat. Nos. 5,695,748 ("the '748 patent"), 5,679,378 ("the '378 patent"), and 5,578,599 ("the '599 patent"), for example, each recite a method of restoring hair growth. The '748 patent recites:

> A process . . . for *restoring hair growth* which comprises the steps of:
>
> (a) applying a cleansing mixture of sage, aloe and nettles to the hair and scalp in an amount and for a period of time sufficient to effect cleansing and then removing same;
>
> (b) applying a treatment mixture of castor oil, shea butter, wheat germ oil and white iodine to the hair and scalp in an amount and for a period of time effective to treat the hair and scalp; and
>
> (c) heating the treatment mixture on the hair and scalp for a period of time sufficient to promote penetration of the treatment mixture into the hair and scalp and then removing the treatment mixture.

'748 patent (Claim 1) (emphasis added). The accompanying [**15] disclosure reveals five-examples in which women and men practiced the claimed method. One "subject's hair began to fill-in in the previously balding and thinning areas and the subject . . . achieved a significant degree of improvement . . . ." *Id.* (Example 3). For another subject, "there [was] a partial filling-in and restoration of the bald spot on the top of the subject's head." *Id.* (Example 4). A third subject noticed that he had "fifty percent more hair in both the frontal and middle sections of his scalp." *Id.* (Example 6).

The '378 patent recites:

> The method for *the restoration of hair growth* . . . which comprises the steps of:

> applying a finite layer of Dead Sea mud to the body surface area to be treated for the restoration of hair growth . . .;
>
> allowing said layer to be undisturbed for a finite time; and
>
> rinsing said layer from said surface area.

'378 patent (Claim 1) (emphasis added). The accompanying disclosure reveals an example in which a man noticed "many sprouts of . . . new hair" after practicing the method for six weeks and ultimately "approximately 25% regrowth over the entire previously bald scalp." *Id.* (Example 1). Another example [**16] discloses the results of a five-month study of men who practiced the invention. In this study, the participants noticed an increase in the number of new hairs on their scalp per month, which varied from 0 to 22. Although [*1359] some participants reported significant growth of hair, there was no evidence that the claimed method resulted in full heads of hair. *See id.* (Example 3).

The '599 patent recites:

> A method for increasing or *restoring hair growth* over the sole administration of a topical minoxidil treatment comprising the concomitant administration of:
>
> a topical preparation of minoxidil in an amount sufficient to promote hair growth, applied to an area of skin where hair growth is to be increased or restored; and
>
> an oral administration of 17 beta-(N-tert-butylcarbamoyl)-4-aza-5-alpha-androst-1-en-3-one in an amount from about 0.05 to about 0.03 mg/Kg to promote hair growth such that hair growth is increased over the administration of minoxidil alone.

'599 patent (Claim 1) (emphasis added). The examples disclosed by the patent show that subjects practicing this method experienced increased growth of hair compared to those using minoxidil alone. Nevertheless, the [**17] patent does not show that this method completely cured baldness by producing a full head of hair.

In light of these disclosures, one of ordinary skill would not construe "restoring hair growth" to mean "returning the user's hair to its original state," as the board required. To the contrary, consistent with Cortright's disclosure and that of other references, one of ordinary skill would construe this phrase as meaning that the claimed method increases the amount of hair grown on the scalp but does not necessarily produce a full head of hair. Properly construed, claim 1 is amply supported by the written description because Example 1 discloses the amount of Bag Balm (R) to apply (about one teaspoon daily) and the amount of time (about one month) in which to expect results. These dosing instructions enable one of ordinary skill to practice the claimed invention without the need for any experimentation. Therefore, we reverse the board's rejection of claim 1.

Claim 15

With respect to claim 15, the examiner made a lack of utility rejection under section 101 because Cortright "offered no proof that such an off-set occurs and has disclosed that this is only speculation." Although [**18] the board purported to reject the examiner's section 101 rejection of claim 15, its new rejection under section 112, P 1 suggests that it did not disagree with the examiner entirely. The board stated that because the written description "merely 'surmises'" that the active ingredient, 8-hydroxy-quinoline sulfate reaches the papilla and offsets the lower levels of male hormone, it did not teach how to use the method of claim 15. It observed further that the written description fails to provide a working example of the subject matter of claim 15 or any evidence that "the effects of lower male hormone levels have been offset [by the claimed method], or even if Bag Balm (R) has reached the papilla." The board also faulted Cortright for not producing evidence that "the resultant hair growth is due to (i) the stimulation of the papilla, and (ii) the offsetting [of] the effects of lower male hormone which is supplied by arteries to the papilla, and not due to some other mechanism(s)." Moreover, it found that the written description indicates that "the underlying basis for the observed physiological phenomenon can not [sic] be predicted from the results obtained," and that this type of [**19] unpredictability alone may "provide a reasonable doubt as to the accuracy of broad statements made in support of the enablement of a claim."

"[HN8]It is not a requirement of patentability that an inventor correctly set forth, or even know, how or why the invention works." *Newman v. Quigg*, 877 F.2d 1575, 1581, 11 U.S.P.Q.2D (BNA) 1340, 1345 (Fed. Cir. 1989); *see also Fromson v. Advance Offset Plate, Inc.*, 720 F.2d 1565, 1570, 219 U.S.P.Q. (BNA) 1137, 1140 (Fed. Cir. 1983) ("It is axiomatic that an inventor need not comprehend the scientific principles on which the practical effectiveness of his invention rests."). Furthermore, statements that a physiological phenomenon was observed are not inherently suspect simply because the underlying basis for the observation cannot be predicted

or explained. Therefore, the board erred in suggesting that Cortright was required to prove the cause of the resultant hair growth.

[*1360] Statements relating to observations that salves applied to the scalp penetrate the skin and reach the papilla or that chemicals affect hormones do not run counter to generally accepted scientific norms. Therefore, a disclosure that the active agent, 8-hydroxyquinoline sulfate, reached the [**20] papilla and offset lower levels of male hormones is not inherently suspect. Nevertheless, we must affirm the rejection of claim 15 because the written description fails to disclose that the active ingredient reaches the papilla or that offsetting occurs. *See In re Bundy*, 642 F.2d 430, 434, 209 U.S.P.Q. (BNA) 48, 51 (CCPA 1981) ("What is necessary to satisfy the how-to-use requirement of § 112 is the disclosure of some activity coupled with knowledge as to the use of this activity."). Here, although the written description states that people observed hair growth after applying Bag Balm (R) to the scalp, it does not disclose that anyone observed the active ingredient reach the papilla and offset the effects of lower levels of male hormones. It states, rather, that "*it is believed* that the rubbed-in ointment offsets the effects of lower levels of male hormones in the papilla and/or provides an antimicrobial effect on infection," and that "Applicant *surmises* that the active antimicrobial agent, 8-hydroxyquinoline sulfate, reaches the papilla, and is effective to off-set the male hormones such as testosterone and/or androsterone, and/or kill or seriously weaken any bacteria about [**21] or in the papilla . . . ." (emphasis added). These statements reflect no actual observations. Moreover, we have not been shown that one of ordinary skill would necessarily conclude from the information expressly disclosed by the written description that the active ingredient reaches the papilla or that off-setting occurs. *See Tronzo v. Biomet, Inc.*, 156 F.3d 1154, 1159, 47 U.S.P.Q.2D (BNA) 1829, 1834 (Fed. Cir. 1998) ("[HN9]In order for a disclosure to be inherent . . . the missing descriptive matter must necessarily be present in the . . . application's specification such that one skilled in the art would recognize such a disclosure."); *see also In re Oelrich*, 666 F.2d 578, 581, 212 U.S.P.Q. (BNA) 323, 326 (CCPA 1981) ("Inherency . . . may not be established by probabilities or possibilities. The mere fact that a certain thing *may* result from a given set of circumstances is not sufficient." (quoting *Hansgirg v. Kemmer*, 26 C.C.P.A. 937, 102 F.2d 212, 214, 40 U.S.P.Q. (BNA) 665, 667 (CCPA 1939)). Therefore, claim 15 does not satisfy the how to use requirement of section 112, P 1.

Conclusion

Accordingly, the decision of the United States Board of Patent Appeals and Interferences is affirmed in part [**22] and reversed in part, and the case is remanded for further proceedings in accordance with this opinion.

COSTS

Each party shall bear its own costs.

AFFIRMED-IN-PART, REVERSED-IN-PART, AND REMANDED

EXPERIMENTAL CELL RESEARCH 224, 39–51 (1996)
ARTICLE NO. 0109

An $\alpha 2\beta 1$ Integrin-Dependent Pinocytic Mechanism Involving Intracellular Vacuole Formation and Coalescence Regulates Capillary Lumen and Tube Formation in Three-Dimensional Collagen Matrix

GEORGE E. DAVIS[1] AND CHARLES W. CAMARILLO

Department of Pathology, Texas A & M University Health Science Center, College Station, Texas 77843-1114

Human endothelial cells, when suspended within three-dimensional collagen matrices, develop intracellular vacuoles that coalesce to form capillary lumens and tubes. Vacuole and lumen formation are completely dependent on the collagen-binding integrin $\alpha 2\beta 1$, while other endothelial cell integrins had no apparent influence. Vacuole formation occurs by a pinocytic process with internalization of plasma membrane and molecules from the extracellular space, such as fluorescent tracers. By immunofluorescence, vacuole membranes were found to contain associated cell surface proteins, proteins involved in endosomal trafficking (i.e., caveolin and annexin II), and F-actin. Furthermore, some vacuole compartments contained von Willebrand factor. Integrin-regulated vacuole formation and coalescence are major mechanisms controlling capillary lumen and tube formation within a three-dimensional extracellular matrix.

INTRODUCTION

The process of angiogenesis, in which new blood vessels sprout from preexisting vessels, appears to be regulated by a variety of molecules which can either stimulate or inhibit the process [1–6]. This process is known to be critical in a variety of cellular processes such as wound healing, development, and cancer [6]. Extracellular matrix (ECM) is known to play a role in angiogenesis and can regulate events such as proliferation, differentiation, migration, and morphogenesis [7–13]. Endothelial cells interact with extracellular matrix through a number of receptors including the integrins [14–16], which have recently been shown to play a role in endothelial cell morphogenetic events *in vitro* and *in vivo* [17–23]. Angiogenic growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) induce angiogenesis *in vivo* and appear to play a major role in the regulation of this process *in vivo* [1, 2, 24, 25]. Some evidence suggests that these two factors can act synergistically to promote endothelial cell morphogenetic events [26, 27]. How integrin-mediated and angiogenic factor-mediated signaling intersect to regulate the angiogenic process is currently unclear.

Many studies have attempted to define different steps in the angiogenic process using *in vitro* models [7–9, 28–31]. In most cases, endothelial cells were exposed to ECM materials in the presence of angiogenic factors. In models utilizing basement membrane matrix, endothelial cells were induced to differentiate into endothelial cord-like structures when exposed to a planar ECM [9, 12, 13]. In other models, endothelial cells were either induced to invade a collagen gel [7] or were resuspended within a three-dimensional collagen gel [31, 32]. In the collagen gel models, capillary-like lumens were demonstrated which closely mimic those observed within collagenous matrix *in vivo* [31]. The molecular mechanisms regulating the formation of endothelial cell lumens in these models is not clear. A study using long-term endothelial cell cultures on plastic surfaces showed that lumen formation is preceded by the formation of intracellular vacuoles that coalesce to form the lumenal compartment [28]. A number of studies *in vitro* and *in vivo* have shown the presence of endothelial cell vacuoles during morphogenetic events [28, 33–38]. The relationship between endothelial vacuoles and lumen formation and the molecular mechanisms regulating capillary lumen formation in three-dimensional ECM environments remains ill-defined.

In this report, we describe experiments using human endothelial cells where, following resuspension in three-dimensional collagen matrices, the cells rapidly develop intracellular vacuoles which progress to capillary lumen-like structures. Endothelial cell vacuoles appear as a result of a pinocytic process which is regulated by the collagen-binding integrin $\alpha 2\beta 1$. Vacuole formation was shown to precede the development of

[1] To whom reprint requests should be addressed at Department of Pathology, Texas A & M University Health Science Center, 208 Reynolds Medical Building, College Station, TX 77843-1114. Fax: (409) 862-1299.

0014-4827/96 $18.00

lumens from individual endothelial cells and groups of endothelial cells. These findings further our understanding of how integrin-mediated endothelial cell interactions with three-dimensional ECM regulate a major morphogenetic event in the angiogenesis process, which is the formation of the characteristic capillary lumen.

MATERIALS AND METHODS

Cells and Tissue Culture

Human umbilical vein and dermal microvascular endothelial cells (Clonetics Corp., San Diego, CA) were propagated as described [13] between passage 2 and passage 10. Human dermal fibroblasts (Clonetics Corp.) and the transformed cell line ECV 304, derived from umbilical vein endothelial cells [39], were grown in DMEM or Medium 199 containing 10% fetal calf serum, respectively.

Antibodies, Reagents, and Chemicals

Monoclonal antibodies known to block integrin function that were utilized are Mab 13 (anti-β1) [40], F17 (anti-α2) [41], P1B5 (anti-α3) [42], Mab 16 (anti-α5) [40], GoH3 (anti-α6) [43], LM 609 (anti-$\alpha v\beta$3) [44], and P1F6 (anti-$\alpha v\beta$5) [45]. Mab 13 and 16 were obtained from Dr. K. Yamada (National Institutes of Health, MD) and F17 was obtained from Dr. H. Gralnick (National Institutes of Health, MD). The other integrin Mabs were obtained from Chemicon Corp. (Temecula, CA). Recombinant bFGF and VEGF were obtained from Upstate Biotechnology (Lake Placid, NY). Rat tail collagen was prepared from adult rat tails as described [46]. Following extraction, the collagen is lyophilized, the dry weight is determined, and then it is resuspended in 0.1% acetic acid at 7.1 mg/ml. Monoclonal antibodies used for immunofluorescent analysis included those directed to PECAM (CD 31) and von Willebrand factor (Dako Corp., Carpinteria, CA); caveolin, paxillin, and focal adhesion kinase (Transduction Laboratories, Lexington, KY); annexin II-light chain, annexin I, annexin IV, and annexin VI (Chemicon, CA); clathrin (ICN, Costa Mesa, CA); cadherin-5 (Immunotech, Westbrook, ME); and α-tubulin, β-tubulin, vimentin, and vinculin (Sigma, St. Louis, MO). Fluorescein–dextran (10,000 Da), carboxyfluorescein, and phalloidin–fluorescein were obtained from Molecular Probes (Eugene, OR).

Endothelial Cell Capillary Lumen Formation in Collagen Gels

Cell preparation. Human endothelial cells are removed from culture plastic flasks using trypsin–EDTA. Trypsin is neutralized with fetal calf serum and the cells are allowed to recover from trypsinization for 20 min in Medium 199 containing 10% serum. The cells are then centrifuged and washed to remove serum and are resuspended in a concentrated form in Medium 199.

Collagen gel preparation. Collagen is added to tubes to which 10× concentrated Medium 199 and NaOH are added in a mixture at 0°C. After thorough mixing, the cells are added at 10^6/ml with a final collagen concentration of 5 mg/ml. The cell–collagen mix is then added at 25 μl per well in 4.5-mm diameter microwells. The collagen is allowed to gel and is equilibrated for 30 min at 37°C in a CO_2 incubator prior to the addition of culture media. In some cases, 1- to 2-μl dots of this mixture were placed onto coverslips. Coverslips were then inverted onto microscope slides with an attached gasket (Nunc, Naperville, IL) which creates eight different wells which were filled with appropriate media.

Cell culture. The serum-free culture medium is Medium 199 containing the reduced-serum II supplement (Upstate Biotechnology), bFGF (40 ng/ml), VEGF (40 ng/ml), phorbol ester (50 ng/ml), and 50 μg/ml of ascorbic acid in a 100-μl volume per 4.5-mm well. Monoclonal antibodies directed to integrin subunits were added at 50 μg/ml in all cases except for the Mab 13 antibody, which was added at 20 μg/ml. Cultures are placed at 37°C in a CO_2 incubator and are examined at different times of culture.

Fixation and analysis. Cultures are fixed with 3% glutaraldehyde in phosphate-buffered saline (PBS) and are evaluated for vacuole or capillary lumen formation. Vacuole formation is quantitated from fixed 3-h cultures. At least 100 cells are evaluated from an individual well for the number of cells with identifiable vacuoles. The cells are evaluated at 200× magnification and are quantitated using an ocular grid. Capillary lumen formation is quantitated from paraffin sections derived from 24- and 72-h fixed cultures. The sections are stained with hematoxylin and eosin. A lumen is counted if the rim of the lumen is covered by endothelial cellular material which occupies more than 75% of the lumenal circumference. Sections are prepared from the paraffin blocks by serial sectioning (4 μm thick) of individual gels. Every fifth section is placed on slides for quantitative analysis. Lumens are quantitated at 200× magnification and the lumenal counts are derived from counting four fields derived from triplicate wells and at least two sections from each gel. Endothelial cell viability was assessed at 3 h of culture by the addition of the tetrazolium dye MTS/PMS (Promega, Madison, WI) at a 1:5 dilution. After 1 h of incubation at 37°C, plates were read at 490 nm in a microplate reader (Bio-Rad, Hercules, CA).

Fluorescence Microscopy

After 3 h of culture, endothelial cells were released from the collagen gels by digesting with 100 μg/ml of bacterial collagenase (Sigma) for 10–15 min at 37°C. The generated cell suspension (3 wells per 22 × 22 mm coverslip) was then seeded onto glass coverslips coated with 20 μg/ml of the synthetic RGD-containing protein Pronectin F (Promega, Madison, WI). Cells are allowed to adhere for 15 min at 37°C. In some cases, cells were surface biotinylated as described [47] except that a membrane-impermeant reagent, sulfosuccinimidobiotin (Pierce, Rockford, IL), was utilized and labeling was performed at 0°C. After washing, the cells were resuspended in collagen and incubated at 37°C for 3 h. The cells were then digested out of the gel and plated on glass coverslips as above. Cells were fixed with 2% paraformaldehyde in PBS for 30 min and, following washing and blocking steps, were incubated with streptavidin–fluorescein at a 1:500 (2 μg/ml) dilution in PBS. Carboxyfluorescein labeling of endothelial cell lumens was performed by the addition of 1 mg/ml of carboxyfluorescein to the culture media for 24 h of a culture that was developing lumens. After 24 h, collagen gels were removed from the microwells with forceps, placed into 35-mm dishes, and washed for 30 min at 37°C with Medium 199 containing no phenol red (two changes of 2 ml each). After washing, the gels were placed onto a microscope slide, a coverslip was gently placed on top of the gel, and the gel was then observed by fluorescence microscopy (Nikon Labphot). Dextran–fluorescein labeling of endothelial cell vacuoles was performed by adding 5 mg/ml of dextran–fluorescein to the culture medium for the first 3 h of culture. The cells were then digested out of the gel and plated onto glass coverslips, as mentioned above, prior to fluorescence microscopy.

Immunofluorescence was performed by harvesting endothelial cells with vacuoles at 3 h of culture and plating them on glass coverslips. The cells were fixed with 2% paraformaldehyde in PBS for 30 min, permeabilized with 0.5% Triton X-100 in PBS, incubated with various monoclonal antibodies in PBS containing 0.1% Triton X-100, 1% goat serum, and 1% bovine serum albumin, and then with a 1:25 dilution of fluorescein-conjugated goat anti-mouse IgG (Dako, Carpinteria, CA) in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin.

Electron Microscopy and Histology

Cultures were fixed with electron microscopy grade 3% glutaraldehyde (Sigma) in PBS. The collagen gels were then processed, embed-

FIG. 2. Capillary network formation in three-dimensional collagen gels. Human umbilical vein endothelial cells were resuspended in collagen gels and were fixed with glutaraldehyde at 72 h of culture. The endothelial cell–collagen gels were then stained with toluidine blue and photographed. A representative field of capillary networks is shown. Arrows indicate endothelial cell nuclei. Bar equals 50 μm.

may be capable of interacting to form cell–cell junctional contacts that are present in the lumenal walls (Fig. 3A). With time, the open lumen structures disappear and the more characteristic closed capillary-like structures are seen (Figs. 4F and 4G). The closed capillary structures coupled with the capillary networks shown above (Figs. 1 and 2) clearly show that networks of capillary tubes develop in this *in vitro* system.

To further address the relationship between endothelial cell vacuole and lumen formation, additional experiments were performed (Fig. 5). A single endothelial cell embedded within a collagen gel was sequentially photographed over a 24-h period (Fig. 5A). Intracellular vacuoles appear, enlarge, and coalesce to form a lumenal compartment. This result suggests that a single cell can form a lumen. Interestingly, in the lowest right panel, a new vacuole became apparent adjacent to the lumen. This vacuole is in between the lumen and a cellular process which was seen extending toward adjacent cells. The vacuole in this location may contribute to the extension of the lumen in the direction of the cell process to facilitate the formation of a capillary network. We have also observed lumen development from two or more adjacent endothelial cells (Fig. 5B). As shown in the figure, a vacuole formed in the cell on the left adjacent to an area of cell–cell contact, while many vacuoles were already present in the cell on the right at the initiation of the photographic sequence. With time the vacuoles coalesce to form a lumenal compartment consisting of two cells. Thus, lumens which form as a result of intercellular contact may also arise through a vacuole formation mechanism.

Control experiments show that vacuolization and lumen formation is not observed when endothelial cells are suspended within methylcellulose under the same media conditions, but is observed when they are resuspended in other matrices such as fibrin (Davis and Camarillo, unpublished observations). Furthermore, minimal vacuole formation occurs when the cells are seeded onto the surface of a collagen gel, showing that three-dimensional ECM contacts markedly enhance the process (not shown). Human dermal microvascular endothelial cells form vacuoles that progress to lumens when suspended within collagen gels while a tumorigenic cell line derived from human umbilical vein endothelial cells, ECV 304, does not develop vacuoles or lumens (Davis and Camarillo, unpublished observations). Human dermal fibroblasts are capable of forming occasional vacuole-like structures following resuspension within collagen gels although they do not progress to lumenal structures (not shown). The percentage of fibroblasts with vacuoles is limited (<10%) compared to greater than 75% of the endothelial cells which develop vacuoles.

The Collagen-Binding Integrin $\alpha 2\beta 1$ Regulates Vacuole and Lumen Formation

To investigate the role of integrins in the formation of capillary lumens within collagen matrices, we added

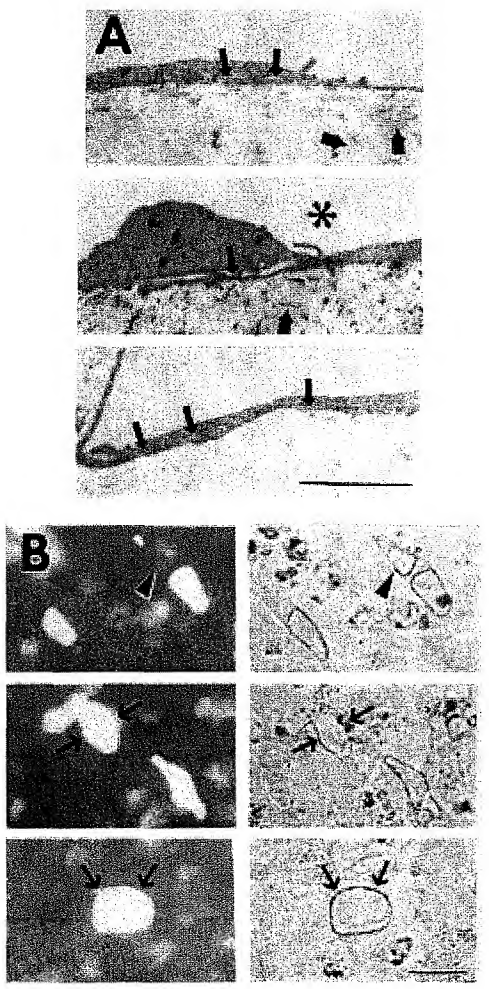


FIG. 3. Presence of endothelial cell–cell junctional contacts and fluorescent labeling of capillary lumens in three-dimensional collagen gels. Human umbilical vein endothelial cells were resuspended in collagen gels, fixed with glutaraldehyde, and processed for electron microscopy. Endothelial cell–cell junctional contacts are shown (A) from cultures fixed at 24 (upper and lower panel) or 120 (middle panel) h. Thin arrows indicate the slit-like spaces between the two interacting endothelial cell processes while thick arrows indicate collagen fibrillar material. The asterisk indicates the lumenal space. Bar equals 2.5 μm. Separate human umbilical vein endothelial cell cultures were prepared in collagen gels and the cultures were placed in medium containing 1 mg/ml of the fluorescent dye 6-carboxyfluorescein (B). After 24 h of culture, the gels were removed from microwells and placed into a 35-mm dish containing Medium 199 to wash out free carboxyfluorescein. After three to four washes over 30 min, the gels were examined under fluorescence microscopy (left) or transmitted light microscopy (right). Arrows indicate lumenal structures that are labeled fluorescently while the arrowhead indicates a lumenal structure that is not labeled. Bar equals 50 μm.

a series of known blocking monoclonal antibodies directed to integrins. Antibodies directed to the β1, α2, α3, α5, α6, $\alpha v\beta$3, and $\alpha v\beta$5 integrin subunits were added to the cultures at doses which markedly inhibit the function of the individual receptors [13]. The integrins $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, α6b1, $\alpha v\beta 3$ and $\alpha v\beta 5$ are expressed by the endothelial cells as we and others have shown previously [13, 14]. After 24 h of culture, the number of capillary lumens was quantitated from par-

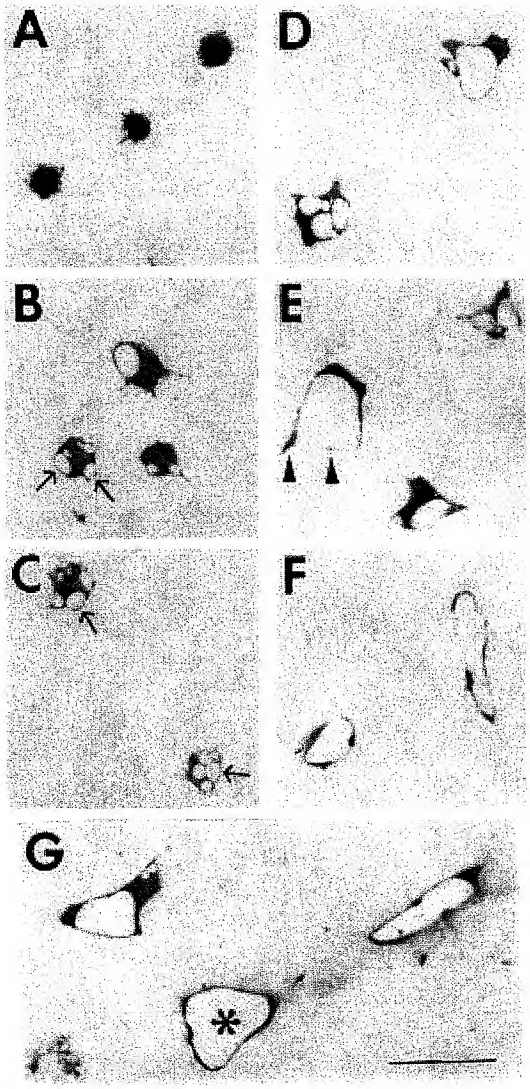


FIG. 4. Time course of capillary formation in three-dimensional collagen gels. Human umbilical vein endothelial cells were resuspended in collagen gels and were fixed at 0 (A), 4 (B), 8 (C), 12 (D), 24 (E), 72 (F), and 120 (G) h of culture with glutaraldehyde and embedded in plastic for electron microscopy. Sections (1.5 μm) were prepared and stained with toluidine blue. Arrows indicate vacuole structures. Arrowheads indicate endothelial cell processes which could participate in junction and capillary network formation. Bar equals 50 μm.

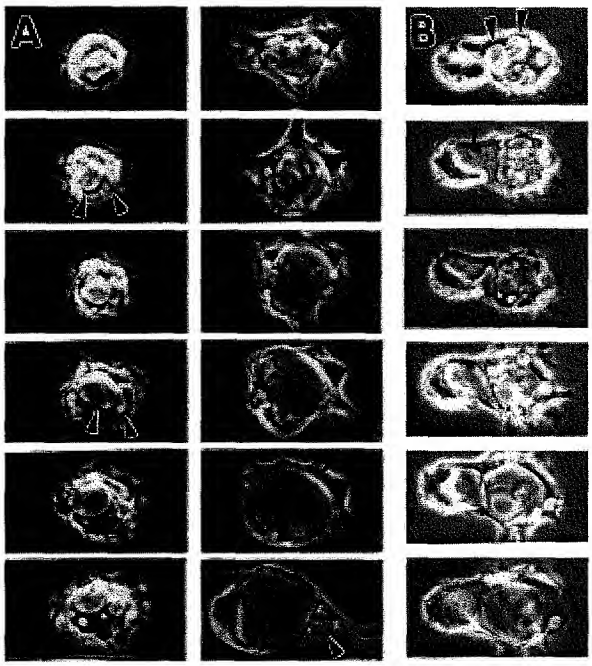


FIG. 5. Development of capillary lumens from endothelial cell intracellular vacuoles. Human umbilical vein endothelial cells were resuspended in collagen gels and 1–2 μl of the cell–collagen mixture was dotted onto coverslips and was allowed to polymerize. The coverslips with the gels facing downward were placed onto a well mounted on a microscope slide. An individual endothelial cell was photographed over time to visualize the lumen formation process (A). Left images, six time points from top to bottom are 1.5, 2.75, 4.25, 6, 7.25, and 8.25 h of culture. Right images, six time points from top to bottom are 9.25, 10.5, 12, 14, 16, and 24 h of culture. Arrowheads indicate endothelial cell vacuoles while arrows delineate the margin of a capillary lumen. The open arrow indicates an endothelial cell process which is out of the plane of focus but is extending to an adjacent group of cells (not seen in panel). Two endothelial cells were photographed in the same manner as above (B). Six time points from top to bottom are 2, 3.5, 4.5, 6, 7, and 9 h of culture. The open arrows indicate the two endothelial cells while the arrowheads indicate endothelial cell vacuoles. Bar equals 50 μm.

affin sections derived from fixed collagen gels. As shown in Fig. 6A, the β1 and α2 subunit antibodies markedly block lumen formation. This same result is observed after 72 h of culture and also occurs if the antibodies are added after 24 h of culture following the formation of early lumens (not shown). The data show that the collagen-binding integrin $\alpha2\beta1$ is a major regulator of capillary formation in a three-dimensional collagen matrix. This result is similar to that shown previously by other investigators [20]. In contrast, the other antibodies had no effect. Interestingly, the $\alpha v\beta3$ integrin, which has recently been shown to be important for angiogenesis in the chick chorioallantoic membrane system [21, 22], had no apparent influence in our *in vitro* system.

In order to investigate the mechanism by which anti-$\alpha2\beta1$ antibodies inhibit lumen formation, experiments were performed to assess whether vacuole formation was affected by the antibodies. As shown in Fig. 6B, antibodies directed to the β1 or α2 integrin subunits

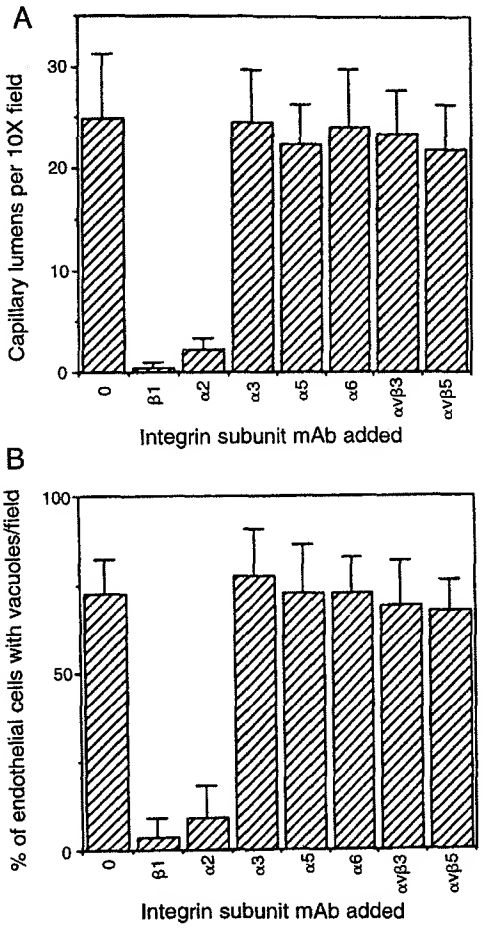


FIG. 6. Influence of anti-integrin blocking antibodies on capillary lumen and endothelial cell vacuole formation in three-dimensional collagen gels. Human umbilical vein endothelial cells were resuspended in collagen gels in the presence or absence of anti-integrin blocking monoclonal antibodies. The antibodies were added at 50 μg/ml except for the anti-β1 subunit antibody which was added at 20 μg/ml. After 24 h of culture, collagen gels were fixed with glutaraldehyde and were processed for light microscopy by embedding the gels in paraffin. Sections of the gels were stained with hematoxylin and eosin and the number of lumens per 10× field was determined for each condition (A). The standard bars represent the average values obtained from triplicate wells and from at least 8 separate fields for each condition. After 3 h of culture, wells were fixed with glutaraldehyde and the percentage of cells with vacuoles was determined for each condition (B). The standard bars represent the average values obtained from triplicate wells and from at least 20 separate fields for each condition.

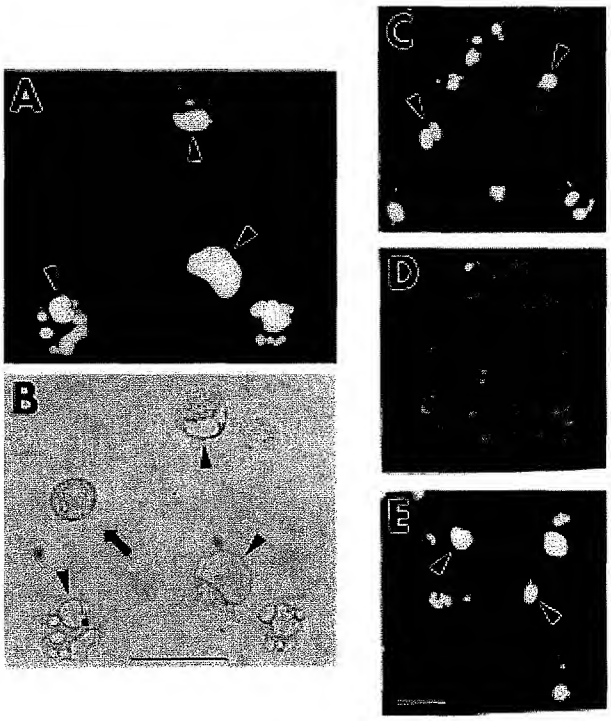


FIG. 8. Dextran-fluorescein labeling of endothelial cell vacuoles and influence of anti-integrin antibodies on fluorescein uptake. Human umbilical vein endothelial cells were resuspended in collagen gels for 3 h of culture with media containing 5 mg/ml of dextran–fluorescein. The cultures were either left untreated (A, B, C) or were treated with 50 μg/ml of anti-integrin antibody directed to the $\alpha 2$ (D) or $\alpha 3$ subunit (E). The cells were then digested out of the gels with bacterial collagenase and were allowed to attach to glass coverslips coated with Pronectin F. The attached cells were then washed several times and were examined by fluorescence (A, C, D, E) or transmitted light microscopy (B). Arrowheads point to endothelial cell vacuoles which are labeled with dextran–fluorescein. The arrow points to a cell without vacuoles which is minimally fluorescently labeled. Bar equals 50 μm.

tion reagent. The cells were then allowed to form vacuoles within collagen gels and, following plating on glass coverslips and fixation, were probed with streptavidin–fluorescein. Endothelial cell vacuole membranes could be found to be labeled, showing that internalization of plasma membrane contributed to the vacuole compartment (Fig. 9A). Cells that were not biotinylated and were treated under the same conditions showed no labeling with streptavidin–fluorescein (not shown).

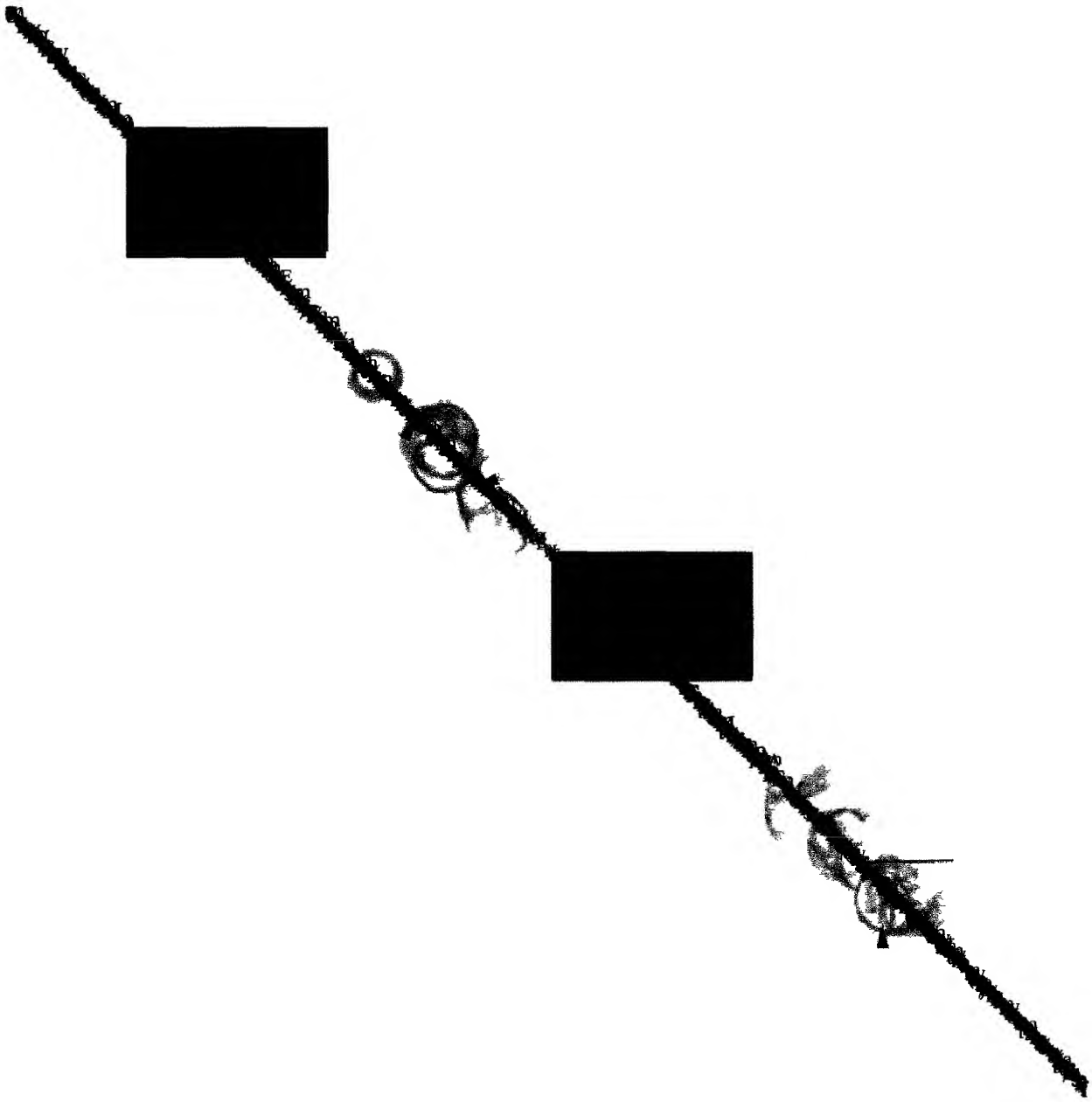
Identification of Proteins Associated with the Vacuole Compartment

To investigate whether known plasma membrane and cytoplasmic proteins are present in the vacuole compartment, vacuolated cells were stained with a variety of monoclonal antibodies and with fluorescein–phalloidin. As shown in Fig. 9, vacuole membranes could be shown to strongly stain with monoclonal antibodies directed to PECAM (CD31), $\beta 1$ integrin subunit, caveolin, and annexin II-light chain and with fluorescein–phalloidin. The presence of F-actin associated with the vacuole membrane is similar to that observed with phagosomes associated with the phagocytic process [51, 52]. Minimal staining was observed with antibodies to clathrin (Fig. 9F), cadherin-5, and annexin IV (not shown). No staining was observed with antibodies directed to α and β tubulin, vimentin, paxillin, focal adhesion kinase, vinculin, annexin I, and annexin VI. Antibodies directed to von Willebrand factor showed some variability but in some cases, it demonstrated marked staining of the inner aspect of the vacuole compartment (Fig. 9H). One possibility is that Weibel-Palade bodies, which contain von Willebrand factor [53], are degranulating into a proportion of the vacuoles. Another possibility is that von Willebrand factor is secreted, polymerized on the endothelial cell surface [54], and pinocytosed into the vacuole compartment. The immunostaining data show that a variety of cell surface proteins are present in the vacuoles supporting the plasma membrane pinocytic mechanism for vacuole formation.

DISCUSSION

In this study, we describe an *in vitro* model system using human endothelial cells to study the regulation of capillary lumen and network formation within three-dimensional collagen matrices. We have found that lumens develop through the formation of intracellular vacuoles that enlarge and coalesce in response to endothelial cell contact with collagen matrix. The endothelial cells develop vacuoles during the first several hours of culture and lumenal structures become evident at 12–16 h of culture. The capillary lumens interconnect within the culture to form extensive capillary networks over a period of days. The collagen-binding integrin $\alpha 2\beta 1$ was found to be required for both the vacuole and lumen formation processes. Vacuole formation occurs through a pinocytic mechanism in that vacuoles can be labeled with fluorescent dyes added to the extracellular medium. Furthermore, labeled plasma membrane was shown to be internalized to the vacuole membrane. This pinocytic mechanism is inhibited by antibodies directed to the $\alpha 2\beta 1$ integrin.

Characterization of the vacuole compartment using immunofluorescent techniques reveals the presence of a number of cell surface and cytoplasmic antigens. Proteins known to be involved in other cellular endocytic events such as caveolin [55–57] and annexin II light chain [58] were found to be vacuole-associated. The vacuole membrane could also be labeled with phalloi-



mechanical force on the collagen matrix, leading to vacuole and capillary network formation rather than collagen gel contraction due to the resistance of the collagen matrix to applied endothelial cell mechanical forces; and (iii) clustering of $\alpha 2\beta 1$ in the plasma membrane to increase cell–matrix binding affinity at selected sites on individual cells while at the same time decreasing adjacent membrane–matrix affinity to initiate the pinocytic process. In this latter case, the higher affinity sites could act as cell–matrix anchor points and vacuoles could develop through invagination of plasma membrane between such anchors.

Since endothelial cells normally are in two-dimensional contact with ECM, the exposure of the cells to three-dimensional matrix appears to induce the cells to reacquire their two-dimensional relationship with ECM. This is accomplished by the production of pinocytic intracellular vacuoles that coalesce to form the new lumenal compartment. These events create a fluid (apical)–matrix (basal) interface that normally exists for endothelial cells. Interesting questions include whether endothelial cell contact with three-dimensional versus two-dimensional collagen matrices induces distinct $\alpha 2\beta 1$-dependent intracellular signals or cytoskeletal associations. A further issue is whether endothelial cell signals derived from $\alpha 2\beta 1$–collagen interactions provide unique signals for this vacuole and lumen formation process. Another possibility is that a number of different integrins may deliver the necessary signals for vacuole and lumen formation. The integrin involved in a particular endothelial cell morphogenetic event may depend on the matrix ligands that are available in the ECM. Preliminary experiments support this idea since endothelial cell vacuole formation in fibrin gels, for example, is not dependent on the $\alpha 2\beta 1$ integrin (Davis, unpublished observations) but appears to be regulated by other integrins.

Much recent attention has been given to the role of the $\alpha v\beta 3$ integrin during angiogenesis *in vivo* [21, 22]. One possibility is that there is a preponderance of Arg-Gly-Asp-containing ligands with affinity for $\alpha v\beta 3$ in the sites where angiogenesis is occurring. Such Arg-Gly-Asp ligands (i.e., denatured collagens, fibrin, vitronectin, osteopontin) [62, 63] would be expected to be particularly enriched in wounded tissues during tissue repair or tumorigenesis, which are major stimuli for angiogenesis [6]. In the above experiments [21, 22], blocking antibodies directed to the $\beta 1$ subunit failed to inhibit angiogenesis, suggesting that $\beta 1$ integrins play a minor role during angiogenesis *in vivo*. These results are difficult to reconcile with our *in vitro* results showing a very strong influence of the $\alpha 2\beta 1$ integrin on capillary morphogenesis. Our results agree more with other studies showing that $\beta 1$ integrin subunit antibodies markedly block early vascular development *in vivo* [18]. This latter developmental system focuses primarily on vasculogenesis. One possibility is that our *in vitro* system may more closely mimic the process of vasculogenesis rather than angiogenesis. As mentioned above, since angiogenesis tends to be associated with wounding and tissue repair while vasculogenesis is a normal developmental process, there are likely to be major differences in the ECM environments during these events. These differences may partially explain why distinct integrins are regulating these unique endothelial cell morphogenetic events.

Control of Capillary Lumen and Tube Formation by Intracellular Vacuole Formation and Coalescence

In this study, single endothelial cells or groups of endothelial cells were shown to develop intracellular vacuoles and progress to form lumens when suspended within collagen gels (see Figs. 4 and 5). Adjacent lumenal structures then connected with each other to form anastomosing networks of capillary tubes (Figs. 1 and 2). Thus, in this system the intracellular vacuole formation process is fundamental to the development of lumens and the formation of capillary tubes. A number of investigators have observed intracellular vacuoles during angiogenic events *in vivo* and *in vitro* and have implicated them in the mechanisms underlying capillary formation [28, 33–38]. For example, vacuoles have been observed at the growing tips of capillary sprouts *in vivo* [33, 34], suggesting a role in lumen formation and extension from preexisting vessels. Our *in vitro* observations coupled with the work of previous investigators strongly support the concept that intracellular vacuole formation is a major mechanism for capillary lumen and tube formation both *in vitro* and *in vivo*. The system that we describe is particularly well suited to study this mechanism of capillary formation.

We have observed that small groups of cells preferentially develop lumens at sites of cell–cell contact (Fig. 5B and Davis and Camarillo, unpublished observations). Several investigators have described this phenomenon as intercellular lumen formation to distinguish it from the intracellular lumen formation mechanism [30, 37, 38]. We believe that intercellular lumen formation is likely mediated by a mechanism similar to that for intracellular lumen formation. At the sites of cell–cell contact there will be minimal ECM between the cells. If one or both of the cells develop invaginations or vacuoles at the cell–cell adhesion site, this would serve to create an ECM-free compartment and preliminary lumen between adjacent cells. The cell–cell adhesive sites may serve as anchors for the pinocytic process much like the cell–ECM contact sites likely do during vacuole formation. It should be pointed out from our work and that of others that capillary lumen formation proceeds through both intracellular and intercellular mechanisms and that these are not

mutually exclusive. In our *in vitro* system, both intracellular and intercellular mechanisms must operate to form lumens and tubes. Furthermore, our work suggests that intracellular and intercellular lumen formation may be mechanistically related.

Our examination of individual cells forming vacuoles and lumens revealed that vacuoles can enlarge over time and likely undergo fusion events with adjacent vacuoles (Fig. 5). Such fusion events may be regulated in a manner similar to other known vesicular fusion events within cells [57, 64, 65]. An important issue is how endothelial cell lumens containing one or more cells connect with adjacent cells to form capillary tubes and vascular networks. Relevant questions are whether network formation involves membrane fusion events between adjacent cells or whether the cells solely interact with each other through cell–cell junctions. In our *in vitro* system, cell–cell junctional contacts are clearly observed (Fig. 3A) which are present in the majority of capillaries *in vivo* [50]. Several reports in the literature describe capillaries *in vivo* that do not appear to contain junctions. These have been referred to as "seamless" capillaries and have been identified during rat brain capillary development and in a variety of other tissues including myocardium, lung, and small intestine [35, 37]. These authors postulate that such seamless capillaries would have to arise through an intracellular lumen formation mechanism.

Endothelial Cell Vacuoles Represent a Novel Pinocytic Compartment Regulating Capillary Lumen Development

It is interesting to consider the relationship between the lumen formation process described here and other endocytic or pinocytic cellular phenomena. The vacuoles that we describe are extremely large, which distinguishes them from endosomes derived from clathrin-dependent and clathrin-independent endocytic pathways [66, 67] or from caveolae [56, 57]. The endothelial cell vacuoles more closely resemble the large pinocytic vesicles that appear during the processes of macropinocytosis [68–70] or phagocytosis [71]. The formation of endothelial cell vacuoles appears to us to be most related to the process of phagocytosis where macrophages and other cells engulf cell attached or floating material from the extracellular space [71]. Phagocytic vacuoles and the endothelial cell vacuoles (Fig. 9D) both strongly stain with fluorescent phalloidin derivatives [52, 71]. Furthermore, macrophage phagocytosis mediated by the Mac-1 (CR3) integrin can be inhibited by both cytochalasins and nocodazole [72]. These two inhibitors completely inhibit the formation of endothelial cell vacuoles (Davis and Camarillo, unpublished observations). During capillary formation, we have occasionally observed that endothelial cellular debris is taken up into endothelial cell vacuoles or lumens. The presence of cellular debris within lumens *in vitro* has been described by a number of investigators [29, 31, 36]. One possibility is that a similar pinocytic mechanism related to how vacuoles and lumens form may allow endothelial cells to clear apoptotic endothelial cells and other debris during blood vessel formation or regression.

The process of phagocytosis is known to proceed from the development of phagocytic vacuoles which then interact with endosomal transport vesicles to cycle proteins from the phagosomes to other cellular compartments [65, 73]. Endothelial cell vacuoles may similarly interact with such transport vesicle pathways. Our current data support this idea in that proteins such as annexin II light chain and caveolin were found to be strongly associated with the vacuole membrane. Annexin II has been found to associate with a variety of endosomal transport pathways [57, 58] and has been implicated in membrane fusion events [58, 64]. Caveolin [74] has been found to associate with caveolae, which are membrane invaginations containing associated signaling molecules and phosphatidylinositol-linked proteins and which participate in vesicular transport [56, 57, 75]. Our immunostaining has revealed that caveolin staining intensity is often increased in areas where vacuoles are in close proximity to each other (Fig. 9F). These data may indicate that caveolin or caveolae could play a role in endothelial cell vacuole formation and fusion events.

Microassay System for Studying Endothelial Cell Vacuole, Lumen, and Tube Formation

The *in vitro* system described in this paper offers some advantages over previous described *in vitro* models of capillary morphogenesis. The microwell and microassay format using 25 μl endothelial cell–collagen gels allows for the rapid examination of many potential culture condition variables. The serum-free culture conditions provide a defined media which can be used to determine with more certainty whether a given media additive directly influences capillary formation. Furthermore, it allows for a dissection of different steps in the capillary development process such as vacuole, lumen, and capillary network formation. Our microassay would also be ideal as a screening method for the identification of new anti-angiogenic agents that address different steps in the angiogenic process.

In this study, we have shown that a major step in capillary morphogenesis, namely the formation of lumens, is regulated by an intracellular vacuole formation and coalescence mechanism that is stimulated by endothelial cell contact with a three-dimensional collagen matrix. The collagen-binding integrin $\alpha 2\beta 1$ is required for the formation of intracellular vacuoles that

progress to capillary lumen-like structures. Vacuole formation occurs secondary to a pinocytic mechanism with internalization of plasma membrane. These data demonstrate that vacuole formation and coalescence are major mechanisms of lumen formation within three-dimensional ECM. The system presented will be useful in future studies to identify specific endothelial cell molecules that are required for vacuole and lumen formation and for the process of angiogenesis.

The authors thank Dr. Kenneth Yamada and Dr. Harvey Gralnick for kindly providing antibodies used in this study. This work was supported by a grant from the American Heart Association, Texas Affiliate (94G-025) to G.E.D.

REFERENCES

1. Klagsbrun, M., and D'Amore, P. A. (1991) *Annu. Rev. Physiol.* 53, 217–239.
2. Folkman, J., and Shing, Y. (1992) *J. Biol. Chem.* 267, 10931–10934.
3. Tolsma, S. S., Volpert, O. V., Good, D. J., Frazier, W. A., Polverini, P. J., and Bouck, N. (1993) *J. Cell Biol.* 122, 497–511.
4. Lane, T. F., Iruela-Arispe, M. L., Johnson, R. S., and Sage, E. H. (1994) *J. Cell Biol.* 125, 929–943.
5. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. (1994) *Cell* 79, 315–328.
6. Folkman, J. (1995) *Nat. Med.* 1, 27–31.
7. Montesano, R., and Orci, L. (1985) *Cell* 42, 469–477.
8. Madri, J. A., and Pratt, B. M. (1986) *J. Histochem. Cytochem.* 34, 85–91.
9. Kubota, Y., Kleinman, H. K., Martin, G. R., and Lawley, T. J. (1988) *J. Cell Biol.* 107, 1589–1598.
10. Ingber, D. E., and Folkman, J. (1989) *Cell* 58, 803–805.
11. Ingber, D. E., and Folkman, J. (1989) *J. Cell Biol.* 109, 317–330.
12. Vernon, R. B., Angello, J. C., Iruelo-Arispe, L., Lane, T. F., and Sage, E. H. (1992) *Lab. Invest.* 66, 536–547.
13. Davis, G. E., and Camarillo, C. W. (1995) *Exp. Cell Res.* 216, 113–123.
14. Albelda, S. M., Daise, M., Levine, E. M., and Buck, C. A. (1989) *J. Clin. Invest.* 83, 1991–2002.
15. Hynes, R. O. (1992) *Cell* 69, 11–25.
16. Schwartz, M. A., and Ingber, D. E. (1994) *Mol. Biol. Cell* 5, 389–393.
17. Bauer, J., Margolis, M., Screiner, C., Edgell, C-J., Azizkhan, J., Lazarowski, E., and Juliano, R. L. (1992) *J. Cell. Physiol.* 153, 437–449.
18. Drake, C. J., Davis, L. A., and Little, C. D. (1992) *Dev. Dyn.* 193, 83–91.
19. Davis, C. M., Danehower, S. C., Laurenza, A., and Molony, J. L. (1993) *J. Cell. Biochem.* 51, 206–218.
20. Gamble, J. R., Matthias, L. J., Meyer, G., Kaur, P., Russ, G., Faull, R., Berndt, M. C., and Vadas, M. A. (1993) *J. Cell Biol.* 121, 931–943.
21. Brooks, P. C., Clark, R. A., and Cheresh, D. A. (1994) *Science* 264, 569–571.
22. Brooks, P. C., Montgomery, A. M. P., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G., and Cheresh, D. A. (1994) *Cell* 79, 1157–1164.
23. Drake, C. J., Cheresh, D. A., and Little, C. D. (1995) *J. Cell Sci.* 108, 2655–2661.
24. Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992) *Nature* 359, 843–848.
25. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrera, N. (1993) *Nature* 362, 841–844.
26. Pepper, M. S., Ferrara, N., Orci, L., and Montesano, R. (1992) *Biochem. Biophys. Res. Commun.* 189, 824–831.
27. Goto, F., Goto, K., Weindel, K., and Folkman, J. (1993) *Lab. Invest.* 69, 508–517.
28. Folkman, J., and Haudenschild, C. (1980) *Nature* 288, 551–556.
29. Maciag, T., Kadish, J., Wilkins, L., Stemerman, M. B., and Weinstein, R. (1982) *J. Cell Biol.* 94, 511–520.
30. Nicosia, R. F., Tchao, R., and Leighton, J. (1982) *In Vitro* 18, 538–549.
31. Montesano, R., Orci, L., and Vassalli, P. (1983) *J. Cell Biol.* 97, 1648–1652.
32. Madri, J. A., Pratt, B. M., and Tucker, A. M. (1988) *J. Cell Biol.* 106, 1375–1384.
33. Speidel, C. C. (1933) *Am. J. Anat.* 52, 1–75.
34. Clark, E. R., and Clark, E. L. (1939) *Am. J. Anat.* 64, 251–301.
35. Wolff, J. R., and Bar, T. (1972) *Brain Res.* 41, 17–24.
36. Dyson, S. E., Jones, D. G., and Kendrick, W. L. (1976) *Cell Tissue Res.* 173, 529–542.
37. Wagner, R. C. (1980) *Adv. Microcirc.* 9, 45–75.
38. Konerding, M. A., van Ackern, C., Steinberg, F., and Streffer, C. (1992) *in* Angiogenesis (Steiner, R., Weisz, P. B., and Langer, R., Eds.), pp. 40–58. Birkhauser Verlag, Basel, Switzerland.
39. Takahashi, K., Sawasaki, Y., Hata, J-I., Mukai, K., and Goto, T. (1990) *In Vitro Cell Dev. Biol.* 25, 265–274.
40. Akiyama, S. K., Yamada, S. S., Chen, W. T., and Yamada, K. M. (1989) *J. Cell Biol.* 109, 863–875.
41. Yamada, K. M., Kennedy, D. W., Yamada, S. S., Gralnick, H., Chen, W-T., and Akiyama, S. K. (1990) *Cancer Res.* 50, 4485–4496.
42. Wayner, E. A., and Carter, W. G. (1987) *J. Cell Biol.* 105, 1873–1884.
43. Sonnenberg, A., Modderman, P. W., and Hogervorst, F. (1988) *Nature* 360, 487–489.
44. Cheresh, D. A., and Spiro, R. C. (1987) *J. Biol. Chem.* 262, 17703–17711.
45. Wayner, E. A., Orlando, R. A., and Cheresh, D. A. (1991) *J. Cell Biol.* 113, 919–929.
46. Bornstein, M. B. (1958) *Lab. Invest.* 7, 134–137.
47. Davis, G. E. (1992) *Exp. Cell Res.* 200, 242–252.
48. Glauret, A. M., and Phillips, R. (1967) *in* Techniques for Electron Microscopy edition (Kay, D. H., ed.), 2nd ed., pp. 213–253. Blackwell, Oxford, UK.
49. Simionescu, N., and Simionescu, M. (1983) *in* Histology: Cell and Tissue Biology (Weiss, L., Ed.), 5th ed., pp. 371–433. Elsevier Biomedical, New York.
50. Cory, A. H., Owen, T. C., Barltrop, J. A., and Cory, J. G. (1991) *Cancer Commun.* 3, 207–212.
51. Reaven, E. P., and Axline, S. G. (1973) *J. Cell Biol.* 59, 12–27.
52. Greenberg, S. (1995) *Trends Cell Biol.* 5, 93–99.
53. Wagner, D. D. (1993) *Thromb. Haemostasis* 70, 105–110.
54. Sporn, L. A., Marder, V. J., and Wagner, D. D. (1986) *Cell* 46, 185–190.

55. Zurzolo, C., Van't Hof, W., van Meer, G., and Rodriguez-Boulan, E. (1994) *EMBO J.* 13, 42–53.
56. Schnitzer, J. E., Oh, P., Pinney, E., and Allard, J. (1994) *J. Cell Biol.* 127, 1217–1232.
57. Schnitzer, J. E., Liu, J., and Oh, P. (1995) *J. Biol. Chem.* 270, 14399–14404.
58. Burgoyne, R. D., and Clague, M. J. (1994) *Trends Biochem. Sci.* 19, 231–232.
59. Berdichevsky, F., Gilbert, C., Shearer, M., and Taylor-Papadimitriou, J. (1992) *J. Cell Sci.* 102, 437–446.
60. Saelman, E. U. M., Keely, P. J., and Santoro, S. A. (1995) *J. Cell Sci.* 108, 3531–3540.
61. Schiro, J. A., Chan, B. M. C., Roswit, W. T., Kassner, P. D., Pentland, A. P., Hemler, M. E., Eisen, A. Z., and Kupper, T. S. (1991) *Cell* 67, 403–410.
62. Davis, G. E. (1992) *Biochem. Biophys. Res. Commun.* 182, 1025–1031.
63. Giachelli, C. M., Liaw, L., Murry, C. E., Schwartz, S. M., and Almeida, M. (1995) *Ann. NY Acad. Sci.* 760, 109–126.
64. Creutz, C. E. (1992) *Science* 258, 924–930.
65. Beron, W., Alvarez-Dominguez, C., Mayorga, L., and Stahl, P. D. (1995) *Trends Cell Biol.* 5, 100–104.
66. Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W., and Schneider, W. J. (1985) *Annu. Rev. Cell Biol.* 1, 1–39.
67. Gruenberg, J., and Howell, K. E. (1989) *Annu. Rev. Cell Biol.* 5, 453–481.
68. West, M. A., Bretscher, M. S., and Watts, C. (1989) *J. Cell Biol.* 109, 2731–2739.
69. Racoosin, E. L., and Swanson, J. A. (1992) *J. Cell Sci.* 102, 867–880.
70. Hewlett, L. J., Prescott, A. R., and Watts, C. (1994) *J. Cell Biol.* 124, 689–703.
71. Greenberg, S., and Silverstein, S. C. (1993) *in* Fundamental Immunology (Paul, W. E., Ed.), pp. 941–964. Raven Press, New York.
72. Swanson, J. A., and Baer, S. C. (1995) *Trends Cell Biol.* 5, 89–93.
73. Desjardins, M., Huber, L. A., Parton, R. G., and Griffiths, G. (1994) *J. Cell Biol.* 124, 677–688.
74. Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y-S., Glenney, J. R., and Anderson, R. G. W. (1992) *Cell* 68, 673–682.
75. Anderson, R. G. W. (1993) *Curr. Opin. Cell Biol.* 5, 647–652.

Received October 24, 1995
Revised version received January 9, 1996

Available online http://breast-cancer-research.com/content/3/2/086

Commentary

Monoclonal antibodies targeting cancer: 'magic bullets' or just the trigger?

Suzanne A Eccles

Institute of Cancer Research, Belmont, Sutton, Surrey, UK

Correspondence: Suzanne A Eccles, Section of Cancer Therapeutics, Institute of Cancer Research, Cotswold Road, Belmont, Sutton, Surrey SM2 5NG, UK. Tel: +44 20 8722 4210; fax: +44 20 8643 0223; e-mail: suzan@icr.ac.uk

Received: 17 August 2000
Revisions requested: 4 September 2000
Revisions received: 27 November 2000
Accepted: 1 December 2000
Published: 20 December 2000

Breast Cancer Res 2001, 3:86–90

(Print ISSN 1465-5411; Online ISSN 1465-542X)

Abstract

The first monoclonal antibodies (mAbs) approved for cancer therapy are now in Phase II and III trials, but the critical mechanism(s) determining efficacy and response in patients are still largely undefined. Both the direct antigen-binding (Fab) and constant (Fc) regions of mAbs can contribute to their biological activity. However, Clynes *et al* (*Nat Med* 2000, 6:443) recently suggested that the latter (at least in experimental models) might be the dominant component in *vivo*, triggering host responses to destroy cancer cells. Those workers showed that in mice lacking 'activation' Fc receptors (FcγRI and FcγRIII), anti-tumour effects of certain mAbs were significantly reduced. In contrast, mice deficient in the 'inhibitory' receptor FcγRIIB responded with tumour growth inhibition and enhanced antibody-dependent cellular cytotoxicity (ADCC). These observations suggest that mAbs might be engineered for preferential binding to FcγRIII to maximise therapeutic benefit. However, further work is needed to establish a definitive cause–effect relationship in experimental models that are more clinically relevant, to determine whether human FcγR isoforms behave in a similar fashion, and to confirm that therapeutic mAbs and host cells can adequately access solid tumour deposits to mediate effective ADCC *in situ*. Finally, the 'cost–benefit' ratio of such modified macromolecules will need to be measured against mini-mAb constructs, antisense oligonucleotides, peptidomimetics and emerging drugs capable of inhibiting key tumour cell signalling pathways.

Keywords: antibody-dependent cellular cytotoxicity, Fc receptors, Herceptin, monoclonal antibody, Rituxan

Introduction

The Holy Grail of cancer therapy is to develop agents capable of selectively destroying disseminated tumour cells while sparing normal tissues. With this aim, major efforts have been directed at harnessing the exquisite specificity of the immune response. Hybridoma technology has enabled the development of tumour selective monoclonal antibodies (mAbs) [1,2], and the past few years have witnessed the approval by the Food and Drug Administration of the first mAbs for the therapy of cancer: Rituxan (anti-CD20) for non-Hodgkin's lymphoma and Herceptin [anti-(c-*erb*B-2/HER-2)] for metastatic breast cancer. The purpose of this commentary is to summarise known and recently reported properties of these mAbs and consider whether recent findings might lead to more effective therapies for cancer.

Targeted therapy for breast cancer

Although the earlier detection of breast cancer and improvements in surgery and adjuvant therapy have

ADCC = antibody-dependent cellular cytotoxicity; bsAbs = bispecific antibodies; CDC = complement-dependent cytotoxicity; EGFR = epidermal growth factor receptor; FcγR = receptors for the Fc region of IgG antibodies; mAb = monoclonal antibody; NK = natural killer; scFv = single-chain antibody variable region.

improved survival rates, there are still around 15,000 deaths in the UK each year and 43,000 in the USA. This is due primarily to the development of drug-resistant metastatic disease. An increasing number of genetic changes have been identified in breast and other cancers, which are now being actively explored for targeted therapy [3]. One of the most exciting new targets is the c-*erb*B-2/HER-2/neu proto-oncogene, which is expressed in 20–30% of breast and other carcinomas. Clinical observations and laboratory experiments have demonstrated convincingly that, together with the related epidermal growth factor receptor (EGFR), it is causally related to maintenance of the malignant phenotype, functioning as a critical signalling molecule in tumour cell proliferation, motility, angiogenesis and metastasis [4]. The accessibility of c-*erb*B-2 at the cell surface, low expression on normal adult tissues and relatively homogeneous distribution within 'positive' tumours and their metastases makes it an ideal candidate for immunotherapeutic intervention [5].

Development of therapeutic mAbs and determination of their mechanisms of action

Initially, attention focused on specificity and affinity, with the selection of mAbs being based primarily on their ability to inhibit tumour cell growth *in vitro*. Some mAbs are extremely potent, with IC_{50} values (concentrations giving half-maximal inhibition) in the nanomolar range, competing well in this regard with low-molecular-mass tyrosine kinase inhibitors. Once good target selectivity had been achieved, mAbs were chemically or genetically modified to decrease their immunogenicity in patients and to improve their physicochemical properties. Antibodies are structurally complex macromolecules with multiple functions. Some, but by no means all, of their activities depend on the complementarity-determining regions within the specific antigen-binding site. When directed against signalling molecules such as CD20, c-*erb*B-2/HER-2 and EGFR, mAbs can exert either agonistic or antagonistic (potentially therapeutic) effects. Simply stated, antagonistic mAbs can be shown to 'remove' and/or to 'switch off' their target antigen, resulting in anti-proliferative effects. For example, 4D5 (the murine mAb from which Herceptin was derived) partly blocks heregulin-induced receptor phosphorylation and transphosphorylation. However, the major effect of these mAbs seems to be receptor downmodulation, potentially preventing heterodimerisation and activation of other HER family members and downstream signalling [6]. Cell cycle progression is inhibited and cells are arrested in G_0/G_1; they can subsequently undergo terminal differentiation or apoptosis, depending on the cell type.

Some antagonistic mAbs preferentially enhance ubiquitination and degradation of their target [7] and yet others (exemplified by certain anti-EGFR mAbs [8]) do not significantly downregulate receptor expression but effectively compete with the cognate growth factors for receptor binding and activation. With Rituxan, it has been shown that the target antigen CD20 is not downregulated, but the mAb induces apoptosis and sensitises cells to the effects of conventional therapy [9]. Thus, even considering the direct effect of mAbs, it is clear that there are a multiplicity of possible responses determined by the properties of the antigen, the antibody, and the cellular context.

Engineering mAbs for improved clinical utility

The major problems of mAb therapy are related to the immunogenicity of rodent proteins and the relatively poor penetration of intact immunoglobulin molecules into solid tumours. The former has been addressed by making chimeric mAbs (human constant region plus mouse variable region) or 'humanised' mAbs in which the human framework Ig contains only rodent sequences encoding the three complementarity-determining regions, as in Herceptin. Another method of reducing immunogenicity and assisting penetration into solid tumours is to remove the constant (Fc) region and to prepare monomeric or dimeric antibody fragments such as Fab, $F(ab')_2$ and single-chain antibody variable region (scFv). However, it was noted that some mAbs were more active *in vivo* than *in vitro*, and this benefit was lost if the Fc portion was removed. Although this is partly explained by the lower affinity and/or shorter half-life of these molecules, results with chemically and genetically modified Fc regions led to an appreciation of the possible contribution of indirect host effects mediated by interactions between IgG Fc and receptors for the Fc region (FcγR) [10].

The recent paper by Clynes *et al* [11] highlights further subtleties relating to mAb interaction with specific FcR subtypes; the authors now suggest that this is a dominant component of the activity of Herceptin and similar mAbs. The experiments described, although elegant, leave several unanswered questions about the interpretation of the data and their clinical relevance. We need to consider whether the differential effects of mAb therapy observed in the genetically modified mice are linked directly to their FcR status, and if so whether similar effects are likely to occur in humans, and finally whether antibody-dependent cellular cytotoxicity (ADCC) is a feasible goal for effective therapy in cancer patients.

The contribution of host effector mechanisms to mAb activity *in vivo*

FcγRs are the key link between humoral and cellular immune responses. They are important in immune regulation and mediate ADCC, endocytosis, phagocytosis, the release of inflammatory cytokines and antigen presentation. FcγR comprise three classes: FcγRI (CD64) FcγRII (CD32) and FcγRIII (CD16). Each class also contains isoforms that exhibit different binding affinities for IgG subclasses, and further complexities arise from their differential expression on host cell populations and the

presence of variant alleles in the low-affinity II and III receptor subtypes [12]. FcγRI and III are multimeric 'activation' receptors, containing both a ligand-binding subunit and a signalling subunit, the immunoreceptor tyrosine-based activation (ITAM) motif, but if co-ligated to the monomeric FcγRIIb (which contains an inhibitory ITIM motif), responses are downregulated.

Herceptin contains a human γ1 Fc and interacts primarily with FcRγIII on natural killer (NK) cells and monocytes. The binding of free mAb is quite weak, but once bound with high affinity to c-*erb*B-2 on the tumour cell surface, it mediates effective ADCC *in vitro* [6]. Clynes *et al* [11] now show that Herceptin (and Rituxan) also bind FcγRIIB (present on monocytes and macrophages, but not NK cells) and that if this interaction is prevented, ADCC is enhanced. They have also shown, with three different experimental systems (including therapy of breast carcinoma xenografts with Herceptin) that the efficacy of mAbs *in vivo* was, first, reduced if the Fc portion was deleted; second, reduced in mice deficient in FcγRI and RIII, and third, enhanced in mice deficient in FcγRIIB. However, first, it should be noted that the breast carcinoma (and lymphoma) were grown ectopically (subcutaneously) and in all cases therapy was commenced on day 0, maximising the opportunity for the administered antibody (or activated host cells) to exert therapeutic effects. Second, the behaviour of mAbs in congenitally athymic mice is not equivalent to that in immunocompetent hosts [13]. In the former, the effector function of NK cells and monocytes is enhanced in compensation for a lack of T cell function, and circulating Ig levels are abnormally low. Although a third model used melanoma cells injected intravenously into inbred C57/bl mice, these are known to be a 'high responder' strain immunologically. On balance, it would therefore be premature to use the current data to predict responses in heterogeneous, generally aged, often immunodeficient, human cancer patients in which the clinical problem is established, disseminated disease.

There are also several extra studies that could strengthen the conclusion that FcR status is causally related to mAb therapeutic efficacy *in vivo*. Although it was shown that the tumours grew similarly in the *nu/nu* hosts and those crossed with different FcγR-deficient strains, it would be important to show the following: (1) that the HER2 expression and kinase activity of the transplanted tumours were equivalent in all hosts, (2) that tumours with different expression levels responded as predicted, and (3) that the tumour response to direct-acting agents was equivalent – these could be HER-2 tyrosine kinase inhibitors, non-ADCC-mediating mAbs, drug or radioisotope conjugates. These studies should exclude any epigenetic modifiers of response that could inadvertently have been introduced by selective breeding. For example, it has been shown that HER-2 expression (and Herceptin) can alter the sensitivity of tumour cells to cytokines such as tumour necrosis factor-α, and it might be that the different hosts vary in their endogenous (or mAb-activatable) cytokine profiles.

With Rituxan, which is a chimeric mAb comprising human γ1 Fc plus mouse anti-CD20 Fab regions, both ADCC and complement-dependent cytotoxicity (CDC) have been demonstrated *in vitro*, although the main determinants of its clinical efficacy have not been defined. Indeed, it has never been formally proved that ADCC operates in patients, and some mAbs that perform well in ADCC assays fail in clinical trials. Clynes's data (confirming reports by Funakoshi [14]) show a significant FcγRIII-dependent host component of anti-CD20 (Rituxan) in the response of xenografts in *athymic* mice. However, in B lymphomas in immunocompetent animals, Tutt *et al* [15] found that in most cases crosslinking and inhibitory signalling by mAbs directed against surface immunoglobulin idiotype, CD19 and CD40 were more important than the recruitment of host effectors.

Thus, although host mechanisms clearly can contribute to mAb-induced therapeutic responses, their importance varies in different situations. Activity *in vitro* (either direct or mediated via ADCC or CDC) does not seem to predict activity *in vivo*, so we must understand that patients' individual responses are the sum of multiple factors including expression of the target antigen (and other signalling molecules that might compensate if the former is inactivated), levels of circulating Ig or immune complexes and the functional status of their effector cells.

How does the manipulation of FcR interactions compare with other strategies?

Host immune responses can also be induced by the use of bispecific antibodies (bsAbs) in which one Fab arm recognises tumour antigen and the other engages epitopes on T cells (CD3) [16], or specific FcγR [17]. These constructs, unlike Herceptin and Rituxan, are monomeric, which might be a disadvantage (because of their lower affinity), but a new class of bsAbs has been designed that recognises tumour cell EpCAM antigen and CD3, and has an Fc composed of a mouse γ2a heavy chain and a rat γ2b heavy chain: like human γ1, two very potent activators of FcR. mAb BiUII has been shown to activate T cells expressing CD3, monocytes and macrophages expressing FcγRIII, NK cells expressing RI, but not B cells expressing RII/CD32 [18]. Although clinical use might be limited by human anti-mouse and anti-rat (HAMA and HARA) responses, these heterologous Fc regions proved more active than either homologous Fc and showed the advantage of recruitment of multiple classes of host effectors. In contrast, bsAbs recognising HER2 and FcγRIII (for example 2B1) have had limited clinical utility owing to toxicity, although bispecific scFv might overcome some of the problems [19]. The relative merits of these different constructs remain to be fully explored clinically [20].

Available online http://breast-cancer-research.com/content/3/2/086

Do the recent observations by Clynes *et al* have implications for the future design of therapeutic mAbs, and how will these measure up to other agents? Theoretically, if the Fc region could be engineered to give selective binding to FcγRIII relative to FcγRIIB, activation of host effector cells could be maximised. This group found that a single amino acid change at residue 265 of the CH2 domain of murine mAb 4D5 was sufficient to reduce its binding to FcR, abrogate ADCC activity and compromise efficacy *in vivo*. However, binding to both the activation RIII and the inhibitory RIIb receptors was decreased, with no evidence of selectivity. The specificity of binding of Ig isotypes to different FcR isoforms and allotypes is complex, with both CH2 regions 234–237 and the CH2/CH3 interface being implicated [21]. In addition, there is a high degree of homology between the ectodomains of many receptors, suggesting very similar binding profiles.

Careful and comprehensive analysis of the binding specificities of mutated mAbs to human effector cells, ideally harvested from cancer patients, would therefore be essential to predict the net effect of changing FcR interactions. FcγRIIB expressed on follicular dendritic cells in germinal centres has also recently been shown to be important for the regulation of B cell recall responses, and it is possible that this could contribute to host anti-tumour responses in patients [22]. A further consideration is the expression of the FcγR variant alleles in control and disease populations, which might also influence response to therapeutic mAbs [23,24].

Many trials with Herceptin are under way, but as previously stated there is as yet no hard evidence that ADCC is contributing to therapeutic response. It would seem logical to explore this further before steps are taken to generate FcγRIII-selective mAbs as proposed by Clynes *et al*, because if patients are unable to mobilise effector cells to sites of metastasis, the strategy will fail. The CAMPATH 1 mAb (which is a rat γ2b isotype directed against CD52) is a powerful inducer of CDC and ADCC against antigen-positive lymphoid malignancies. However, although it depleted target cells effectively from blood, spleen and bone marrow, it was much less effective against solid tumour deposits [25].

Increasingly, emphasis is being placed on pharmacodynamic endpoints and surrogate markers of response in trials of novel therapies, and mAbs should be no exception. It should be possible to check that patients have adequate levels of NK cells and monocytes before therapy and that these are responsive in ADCC assays. Once therapy is under way, trials could be designed (similar to those with bsAbs) to assess immunological function, for example evidence of CD16-positive host cell recruitment into accessible tumour deposits and circulating cytokine levels. In addition, to examine whether other mechanisms are operating, it would be worth checking for the induction of idiotypic antibody cascades and cell-mediated responses to HER-2.

Finally, the efficacy and cost of novel engineered mAbs must be critically compared with new low-molecular-mass agents that inhibit c-*erb*B-2 and EGFR tyrosine kinases. These include a 1.5 kDa exocyclic anti-HER2/neu peptide mimic [26] and also orally active agents with cellular inhibitory activity in the nanomolar range [27]. Whatever the final outcome, the use of increasingly sophisticated genetically engineered antibodies, cells and animal models will lead to a much greater understanding of tumour–host interactions and how they can best be manipulated for therapeutic benefit. Even when tumours are preselected to be antigen positive, only a minority of patients respond to mAb therapy. If Clynes *et al* are correct, this might be due to the ability of their NK cells and monocytes to become 'triggered' by the therapeutic mAb to kill tumour cells. These are exciting times and effective targeted therapy for cancers is close to being a reality. Whether the 'magic bullets' will be mAbs, their derivatives or synthetic drugs remains to be seen.

References

1. Green MC, Murray JL, Hotobagyi GN: **Monoclonal antibody therapy for solid tumors.** *Cancer Treat Rev* 2000, 26:269–286.
2. Weiner LM: **Monoclonal antibody therapy for cancer.** *Semin Oncol* 1999, 26(suppl. 14):43–51.
3. Gelmon KA, Eisenhauer EA, Harris AL, Ratain MJ, Workman P: **Anticancer agents targeting signalling molecules and cancer cell environment: challenges for drug development?** *J Natl Cancer Inst* 1999, 91:1281–1287.
4. Hynes N: **Tyrosine kinase signalling in breast cancer.** *Breast Cancer Res* 2000, 2:154–157.
5. Eccles S: **c-*erb*B-2 as a target for immunotherapy.** *Expert Opin Invest Drugs* 1998, 7:1879–1896.
6. Sliwkowski MX, Lofgren JA, Lewis GD, Hotaling TE, Fendly BM, Fox JA: **Nonclinical studies addressing the mechanism of action of action of Trastuzumab (Herceptin).** *Semin Oncol* 1999 26 (suppl 12):60–70.
7. Klapper LN, Waterman H, Sela M, Yarden Y: **Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2.** *Cancer Res* 2000, 60: 3384–3388.
8. Modjtahedi H, Dean CJ: **Antibody-induced inhibition of growth of EGFR overexpressing tumours occurs in the absence of receptor down-regulation.** *Int J Oncol* 1995, 7:783–788.
9. Maloney DG: **Preclinical and Phase I and II trials of Rituximab.** *Semin Oncol* 1999, 26(suppl. 14):74–78.
10. Modjtahedi H, Eccles S, Sandle J, Box G, Titley J, Dean C: **Differentiation or immune destruction: two pathways for therapy of squamous cell carcinomas with antibodies to the epidermal growth factor receptor.** *Cancer Res* 1994, 54:1695–1701.
11. Clynes RA, Towers TL, Presta LG, Ravetch JV: **Inhibitory Fc receptors modulate *in vivo* cytotoxicity against tumor targets.** *Nat Med* 2000, 6:443–446.
12. Gessner JF, Heiken IL, Tamm A, Schmidt RE: **The IgG Fc receptor family.** *Ann Hematol* 1998, 76:231–248.
13. Eccles SA, Purvies HP, Styles JM, Hobbs SM, Dean CJ: **Pharmacokinetic studies of radiolabelled rat monoclonal antibodies recognising syngeneic sarcoma antigens: II. Effect of age and host immune status.** *Cancer Immunol Immunother* 1989, 30: 13–20.
14. Funakoshi S, Longo DL, Murphy WJ: **Differential *in vitro* and *in vivo* antitumour effects mediated by anti-CD40 and anti-CD20 monoclonal antibodies against human B cell lymphomas.** *J Immunother* 1996, 19:93–101.

15. Tutt AL, French RR, Illidge TM: Monoclonal antibody therapy of B cell lymphoma: signaling activity on tumor cells appears more important than recruitment of effectors. *J Immunol* 1998, 161:3176–3185.
16. Zhu Z, Lewis G, Carter P: Engineering high affinity humanised anti-p185^{HER2}/anti-CD3 bispecific F(ab')$_2$ for efficient lysis of p185^{HER2} overexpressing tumor cells. *Int J Cancer* 1996, 74: 185–192.
17. Watanabe M, Wallace PK, Keler T: Antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent cellular cytotoxicity (ADCC) of breast cancer cells mediated by bispecific antibody MDX-210. *Breast Cancer Res Treat* 1999, 53:199–207.
18. Zeidler R, Mysliwietz J, Csánady M: The Fc-region of a new class of intact bispecific antibody mediates activation of accessory cells and NK cells and induces direct phagocytosis of tumor cells. *Br J Cancer* 2000, 83:261–266.
19. McCall AM, Adams GP, Amoroso AR, Nielsen UB, Zhang L, Horak E, Simmons H, Schier R, Marks JD, Weiner LM: Isolation and characterisation of an anti-CD16 single chain Fv fragment and construction of an anti-HER2/neu/anti-CD16 bispecific scFv that triggers CD16- dependent tumor cytolysis. *Mol Immunol* 1999, 36:433–445.
20. Hadden JW: The immunology and immunotherapy of breast cancer: an update. *Int J Pharmacol* 1999, 21:79–101.
21. Wines BD, Powell MS, Parren PWHI, Barnes N, Hogarth PM: The IgG Fc contains distinct Fc receptor (FcR) binding sites: the leukocyte receptors FcγR1 and FcγRIIa bind to a region in the Fc distinct from that recognised by neonatal FcR and protein A. *J Immunol* 2000, 164:5313–5318.
22. Qin D, Wu J, Vora KA: Fcγ receptor IIB on follicular dendritic cells regulates the B cell recall response. *J Immunol* 2000, 164:6268–6275.
23. Lehmbecher T, Foster CB, Zhu S: Variant genotypes of the low affinity Fcγ receptors in two control populations and a review of low-affinity Fcγ receptor polymorphisms in control and disease populations. *Blood* 1999, 94:4220–4232.
24. Callahan MB, Le Baccon P, Mossuz P: The IgG Fc receptor FcγRIIB is a target for deregulation by chromosomal translocation in malignant lymphoma. *Proc Natl Acad Sci USA* 2000, 97:309–314.
25. Dyer M: The role of CAMPATH-1 antibodies in the treatment of lymphoid malignancies. *Semin Oncol* 1999, 26:52–57.
26. Park B-W, Zhang H-T, Wu C: Rationally designed anti-HER-2/neu peptide mimetic disables P185$^{HER2/neu}$ tyrosine kinases *in vitro* and *in vivo*. *Nat Biotechnol* 2000 18:194–198.
27. Noonberg SB, Benz CC: Tyrosine kinase inhibitors targeted to the epidermal growth factor receptor subfamily: role as anticancer agents. *Drugs* 2000 59:753–767.

Thursday, Feb 26, 2004

FDA Approves Avastin, A Targeted Therapy for First-Line Metastatic Colorectal Cancer Patients

SOUTH SAN FRANCISCO, Calif. -- February 26, 2004 -- Genentech, Inc. (NYSE: DNA) announced today that the U.S. Food and Drug Administration (FDA) has approved Avastin™ (bevacizumab) to be used in combination with intravenous 5-Fluorouracil-based chemotherapy as a treatment for patients with first-line-or previously untreated-metastatic cancer of the colon or rectum. Avastin is the first FDA-approved therapy designed to inhibit angiogenesis, the process by which new blood vessels develop, which is necessary to support tumor growth and metastasis. Genentech will begin shipping Avastin within three calendar days.

The Avastin FDA approval is based on data from two trials. The pivotal trial was a large, placebo-controlled, randomized study that demonstrated a prolongation in the median survival of patients treated with Avastin plus the IFL (5-FU/Leucovorin/CPT-11) chemotherapy regimen by approximately five months, compared to patients treated with the IFL chemotherapy regimen alone (20.3 months versus 15.6 months). In addition, this study demonstrated an improvement in progression-free survival (PFS) of more than four months (10.6 months in the Avastin/IFL arm compared to 6.4 months in the IFL-alone arm). The survival and PFS results observed when Avastin is added to first-line chemotherapy are the longest ever reported in a randomized, Phase III study of patients with metastatic colorectal cancer.

In the pivotal trial, the most serious adverse events that occurred with Avastin included gastrointestinal perforations and wound healing complications, which were uncommon. The most common severe adverse events in this trial included hypertension, which was managed with oral medications, nosebleeds and asymptomatic proteinuria. Adverse events observed in other trials with Avastin included hemorrhage, congestive heart failure and thromboembolism.

"Today marks an important shift in the treatment of metastatic colorectal cancer, with the approval of an innovative treatment based on elegant science that targets cancer in an entirely new way," said Arthur D. Levinson, Ph.D., Genentech's chairman and chief executive officer. "The FDA's approval of Avastin would not have been possible without the dedication and passion of hundreds of

Genentech employees, clinical trial investigators, patient advocates, the FDA and, most importantly, all of the colorectal cancer patients and their families who volunteered for Avastin clinical trials. We're pleased that patients diagnosed with metastatic colorectal cancer today have a new treatment option."

"When I was diagnosed with Stage Four colorectal cancer, my first thought was of my family and whether there were any treatments that could help me," said Earl Woodard, a commercial airline pilot from Carthage, N.C. "I received Avastin in the Phase III clinical trial. I am not only excited to have benefited from Avastin and chemotherapy, but it is also a great feeling to have participated in a clinical trial that has led to a new drug being approved for patients with metastatic colorectal cancer."

About the Avastin Filing

The Avastin filing was submitted under the FDA's Fast Track program, which permits submission of documents on an ongoing-or "rolling"-basis to facilitate the review process. Genentech submitted the final documents for the Avastin Biologics License Application (BLA), which contained data from more than 1,400 patients who received treatment with Avastin in clinical trials, in September 2003. In November 2003, the FDA granted Priority Review status for Avastin and committed to reviewing the submission within six months of filing.

"Every nine minutes someone in the United States dies of colorectal cancer. As a patient advocate, I understand the desperate need for new therapies for patients with this disease," said Kevin Lewis, board chairperson of the Colon Cancer Alliance.

About the Avastin Pivotal Trial

The Avastin pivotal trial enrolled 925 patients with first-line (previously untreated) metastatic colorectal cancer, which is cancer that has spread beyond the colon or rectum. This trial was designed with a primary endpoint of survival and compared survival of patients treated with Avastin plus the IFL chemotherapy regimen to those treated with IFL chemotherapy and placebo. In addition to showing an improvement in survival in all patient populations studied, the trial also met its secondary endpoints by improving progression-free survival, response rate and duration of response.

About VEGF and Tumor Angiogenesis

The link between angiogenesis and cancer growth has been discussed by many researchers for decades. It wasn't until 1989 that a key growth factor influencing the process, Vascular Endothelial Growth Factor (VEGF), was discovered by Napoleone Ferrara, M.D., a staff scientist at Genentech. Dr. Ferrara and his team cloned VEGF, providing some of the first evidence that a specific angiogenic growth factor existed. This research was published in the journal Science in 1989. Dr. Ferrara then created a mouse antibody to this

protein. In 1993, Dr. Ferrara and his team at Genentech, in a study published in Nature, demonstrated that the antibody directed against VEGF could suppress angiogenesis and tumor growth in preclinical models, providing compelling evidence that VEGF can play a critical role in tumor growth. Clinical studies with a humanized version of the antibody, Avastin, began in 1997.

"Since the pivotal trial results were presented last year, I have had the privilege of meeting several patients who have received treatment with Avastin, and this has been the most rewarding part of watching a scientific theory progress from the lab to the clinic," said Dr. Ferrara. "The approval of Avastin is a testament to the many scientists both within Genentech and around the world who have worked tirelessly, even in the face of adversity and skepticism, to contribute to our understanding of angiogenesis and VEGF."

"Dr. Ferrara's scientific accomplishments and the approval of Avastin mark a turning point in science as it proves the long-pursued angiogenic hypothesis and, through an elegantly designed clinical trial, has turned a theory into a treatment for metastatic colorectal cancer patients," said Judah Folkman, M.D., professor of pediatric surgery at Children's Hospital and Harvard Medical School.

About Avastin

Avastin is a therapeutic antibody designed to inhibit VEGF, a protein that plays an important role in tumor angiogenesis and maintenance of existing tumor vessels. By inhibiting VEGF, Avastin is designed to interfere with the blood supply to a tumor, a process that is critical to a tumor's growth and metastasis. For full prescribing information and boxed warnings on Avastin and information about angiogenesis, visit http://www.gene.com. For more information on Avastin, visit www.avastin.com.

Based on data showing that VEGF may play a broad role in a range of cancers, Genentech is pursuing a late-stage clinical development program with Avastin evaluating its potential use in metastatic colorectal, renal cell (kidney), breast and non-small cell lung cancers. Avastin is also being evaluated in earlier stage trials as a potential therapy in prostate, ovarian, melanoma and several types of solid tumor cancers and hematologic malignancies.

About Colorectal Cancer

According to the American Cancer Society, more than 150 patients die every day from colorectal cancer in the United States. Colorectal cancer is the second leading cause of cancer death in the United States, the third most frequently diagnosed cancer, and approximately 147,000 new cases of colorectal cancer will be diagnosed in the United States in 2004.

About Genentech BioOncology

Genentech is committed to fundamentally changing the way cancer is

treated by establishing a broad oncology portfolio of innovative, targeted therapies that can improve patients' lives. Led by Rituxan® (Rituximab) and Herceptin® (Trastuzumab), the first two therapeutic antibodies approved to treat cancer in the United States and Avastin™ (bevacizumab), the first anti-angiogenic therapy approved to treat cancer in the United States, the BioOncology portfolio includes marketed and pipeline products in clinical trials for the seven most common lethal cancers.

Genentech has a robust pipeline of potential oncology therapies, including a small molecule designed to target the human epidermal growth factor receptor (HER1) pathway (also known as EGFR) and a therapeutic antibody directed at the HER pathway. To broaden Genentech's portfolio of targeted cancer therapies, research programs are leveraging Genentech's expertise in targeting additional components of the HER and angiogenesis pathways, as well as pathways that instruct cancer cells to die (i.e., apoptosis), and B-cell oncology.

About Genentech

Genentech is a leading biotechnology company that discovers, develops, manufactures and commercializes biotherapeutics for significant unmet medical needs. Eighteen of the currently approved biotechnology products originated from or are based on Genentech science. Genentech manufactures and commercializes 13 biotechnology products in the United States. The company has headquarters in South San Francisco, California and is traded on the New York Stock Exchange under the symbol DNA. For additional information about the company, please visit http://www.gene.com.

Genentech will be offering a live webcast of a discussion by Genentech management on Thursday, February 26, 2004 at 3:00 p.m. PT. The live webcast may be accessed on Genentech's website at http://www.gene.com. An archive of this webcast will be available until 5:00 p.m. PT on March 4, 2004. An audio replay of the webcast will be available beginning at 6:00 p.m. PT on February 26, 2004, through 5:00 p.m. PT on March 4, 2004. Access numbers for this replay are: 1-800-642-1687 (US/Canada) and 1-706-645-9291 (international); conference identification number is 5855709.

# # #

For full prescribing information for Avastin, please call 650-225-7739 or visit http://www.gene.com.